Functional characterization of PHHI-inducing mutations in SUR1

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Wael Tawakkol

aus Kairo, Ägypten

1 Referent: Prof. Dr. med. Dr. rer. nat. habil. M. Schwanstecher
2 Referent: Prof. Dr. rer. nat. habil. L. Beerhues
eingereicht am: 23.08.2001
mündliche Prüfung am: 24.10.2001

The following parts of the thesis have been published after approval of the Faculty of Science, represented by the supervisor :

Congress contributions:

TAWAKKOL W and SCHWANSTECHER M: Loss of potassium channel opener binding is indicative for PHHI-mutations in SUR1. *Naunyn Schmiedeberg ´s Arch Pharmacol 361 (suppl.)*, 4, R 77; 41. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie, Mainz, March 2000.

TAWAKKOL W and SCHWANSTECHER M: Loss of potassium channel opener binding is indicative for PHHI-mutations in SUR1. *Exp. Clin Endocrinol Diabetes 108 (suppl.)*, pDo072; 35. Jahrestagung der Deutschen Diabetes-Gesellschaft, München, May - June 2000.

BEYER M, TAWAKKOL W, HECHT HJ and SCHWANSTECHER M: Tertiärstruktur der Nukleotid-bindenden Regionen in SURs. *Naunyn Schmiedeberg ´s Arch Pharmacol 361 (suppl.)*, 4, R 73; 42. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie, Mainz, March 2001.

BEYER M, TAWAKKOL W, HECHT HJ and SCHWANSTECHER M: Tertiärstruktur der Nukleotid-bindenden Regionen in SURs. *Diabetes und Stoffwechsel 10 (suppl.)*, 30; 36. Jahrestagung der Deutschen Diabetes Gesellschaft, Aachen, May 2001.

The work described here was done in the period from April 1997 to July 2001 at the “Institut für Pharmakologie und Toxikologie der Technischen Universität Carolo-Wilhelmina zu Braunschweig”.

I would like to express my great gratitude to Prof. Dr. med. Dr. rer. nat. habil. M. Schwanstecher for the kind supervision of my thesis. Prof. M. Schwanstecher has been very helpful and his encouragement, support, valuable guidance and criticism were the basis for the realization of this work. I would also like to thank PD Dr. med. Christina Schwanstecher for her invaluable help during preparation of the manuscript.

I wish to express my sincere gratitude to Dr. HJ Hecht for his professional cooperation with structure modeling.

It gives me a great pleasure to express my thanks to Mrs U. Herbort-Brand, Mrs S. Warmbold, Mrs G. Müller, Mrs H. Fürstenberg, Mrs B. Pieper (molecular biology and cell culture) and Mrs G. Wittenberg, Mrs V Lier-Glaubitz (binding studies) for their excellent technical assistance.

I also want to express my thanks to all co-workers of the institute for their readiness to help, the fruitful discussions which contributed to the success of the work and for the wonderful working atmosphere which was positively reflected on the smooth running of the work.

I am greatly indepted to my wife for her encouragement, help and useful comments during the whole period of the work.

To Hanan

List of contents

List of abbreviations **V**

1. Introduction **1**

1.1. ATP-sensitive potassium channels 1

1.1.1. *K_{ATP} channels in pancreatic B-cells* 1

1.1.2. *K_{ATP} channels in neurons* 2

1.1.3. *K_{ATP} channels in heart and skeletal muscle* 2

1.1.4. *K_{ATP} channels in smooth muscle* 3

1.1.5. *Structure of K_{ATP} channels* 3

1.2. Sulfonylurea receptors 4

1.2.1. *Sulfonylurea receptors are ABC- proteins* 5

1.2.2. *Cloning of sulfonylurea receptors* 8

1.2.3. *Mutations in SUR1 and K_{IR}6.2 cause PHHI* 8

1.2.3.1. *PHHI results as a consequence of reduced open probability of SUR1/K_{IR}6.2 channels* 8

1.2.3.2. *Incidence and clinical presentation of PHHI* 9

1.3. Modulation of K_{ATP} channel activity 9

1.3.1. *Cytosolic nucleotides* 9

1.3.2. *Sulfonylureas* 10

1.3.2.1. *Structure* 10

1.3.2.2. *Mechanism of action* 11

1.3.3. *Potassium channel openers* 12

1.3.3.1 *Introduction* 12

1.3.3.1.1. *Diazoxide* 13

1.3.3.1.2. *Pinacidil* 13

1.3.3.1.3. *Other potassium channel openers* 14

1.3.3.2. *Therapeutic potential* 14

1.3.3.3. *Identification of the receptor binding sites for KCOs* 15

2. Aims of the study **16**

3. Materials and Methods **17**

3.1. Cultivation, passaging and perservation of COS7 cells 17

	3.1.1.	*Solutions and media for cell culture*	17
	3.1.2.	*Cultivation of COS7-cells*	18
	3.1.3.	*Passaging of COS7-cells*	18
	3.1.4.	C*ryopreservation of COS7-cells*	19
3.2.	Prepartion of mutations		19
	3.2.1.	*Solutions for mutagenesis*	19
	3.2.2.	*QuikChange site-directed mutagenesis*	20
	3.2.3.	*Digestion of the products*	20
3.3.	Transformation and plasmid preparation		20
	3.3.1.	*Solutions*	20
	3.3.2.	*Production of transformation competent cells*	23
	3.3.3.	*Transformation*	24
	3.3.4.	*Plasmid preparation*	24
	3.3.5.	*Sequencing*	25
3.4.	Transfection		25
	3.4.1.	*Solutions for transfection*	25
	3.4.2.	*Transfection by means of the DEAE dextran method*	27
3.5.	Membrane preparation		27
	3.5.1.	*Solutions*	27
	3.5.2	*Preparation of membranes from COS7-cells*	28
3.6.	Protein determination		29
	3.6.1.	*Solutions*	29
	3.6.2.	*Procedure*	29
3.7.	Binding experiments with COS membranes		29
	3.7.1.	*Solutions*	29
	3.7.1.1.	*Stock solution of radio ligand*	29
	3.7.1.2.	*Stock solutions of test substances*	30
	3.7.2.	*Preparation of membranes*	30
	3.7.3	*Binding experiments*	30
	3.7.3.1.	*Principle of the filtration assay*	30
	3.7.3.2.	*Incubation*	31
	3.7.3.3.	*Filtration and scintillation counting*	31
3.8.	Modeling		31
3.9.	Data evaluation		32

3.9.1. *Statistics* 32
3.9.2. *Specific binding* 32
3.9.3. *Equilibrium binding* 32
3.9.4. *Dissociation constants* 32
3.9.5. *Determination of diazoxide binding to SUR1* 33
3.9.6. *Determination of ATP-induced inhibition of glibenclamide binding* 33
3.9.7 *Detemination of B_{max}* 33

4. Results 34
4.1. Application of the “QuikChange site-directed mutagenesesis” method 34
4.1.1. *Principle of the method* 34
4.1.2. *Design of the mutagenic oligonucleotide primers* 36
4.2. Functional consequences of point mutations in SUR1 known to induce PHHI 38
4.2.1. *Analysis of high affinity [^{3}H]glibenclamide binding* 39
4.2.2. *PHHI mutations with complete loss of diazoxide binding* 40
4.2.3. *PHHI mutations with partial loss of diazoxide binding* 41
4.2.4. *Mutations that do not affect diazoxide binding* 41
4.2.5. *Correlation of diazoxide binding with the effect of ATP on glibenclamide binding* 42
4.3. Homology modeling of the nucleotide binding folds in SUR1 43
4.3.1. *Comparison between the sequences of HisP and the NBFs of SUR1* 43
4.3.2. *Analogous model for the tertiary structure of NBF1 and NBF2 of SUR1* 45
4.3.2.1. *Interaction of NBF2 with ATP* 47
4.3.2.2. *Interaction of NBF1 with ATP* 48
4.3.2.3. *PHHI mutations in NBF2* 51

5. Discussion 52
5.1. Loss of of KCO-binding is indicative for PHHI 52
5.2. The tertiary structure of the NBFs of SURs closely resembles that of HisP 56
5.3. The tertiary structure of NBF2 in SUR1 predicts the functional consequences of PHHI mutations 57

6 Summary 58

7. References 60

List of abbreviations

ADP	adenosine-5´-diphosphate
ATP	adenosine-5´-triphosphate
bidist.	bidistilled
B_{max}	number of binding sites
BSA	bovine serum albumin
CFTR	Cystic Fibrosis transmembrane conductance regulator
COS	CV1, Origin of SV40 (s. 3.1.2.)
DEAE	diethylaminoethanol
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calf serum
Fig.	figure
g	gravity (9,81 m/sec^2)
^{3}H	tritium (isotop of hydrogen molecule)
HBSS	HEPES buffered saline solution
HEPES	[4-(2-hydroxyethyl)-piperazino]-ethanesulfonic acid
HisP	histidine permease
HIT	hamster insulin-secreting tumor cell line
IC_{50}	half-maximally inhibitory concentration
KCO	K^+-channel opener (potassium-channel-opener)
K_{ATP}-channal	ATP-sensitive potassium channel
K_D	dissociation constant
K_{IR}	inwardly-rectifying potassium channel
NBF	nucleotide binding fold
p	probability
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PHHI	persistent hypoglycemic hyperinsulinemia of infancy
PMSF	phenylmethylsufonylfluoride
rpm	rotation per minute
SEM	standard average deviation
SUR	sulfonylurea receptor
TAE	tris-acetate-EDTA
TBS	tris buffered saline
TMDI	transmembrane domain I
TMDII	transmembrane domain II
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
wt	wild type

1. Introduction

1.1. ATP-sensitive potassium channels

ATP-sensitive potassium channels (K_{ATP} channels) are found in different cell types: in pancreatic B-cells, in neurons, in heart, in skeletal muscle cells and in smooth vessel muscle cells (AGUILAR-BRYAN and BRYAN, 1999). In all these cells, K_{ATP} channels are important for coupling the metabolic status to electrical activity (NOMA, 1983; MISLER et al., 1986; ASHCROFT, 1988). The activity of the channels is reduced by ATP and increased by MgADP. In pancreatic B-cells, the opening probability of the channels determines the resting membrane potential. Thus, the channels serve as a link between glucose-induced metabolic changes and electrical activity and are of central importance for stimulus secretion coupling (PANTEN et al. 1992; AGUILAR-BRYAN and BRYAN, 1999). In the heart and in neurons, K_{ATP} channels could be involved in regulation of electrical activity in case of ischaemia (GROSS and AUCHAMPACH, 1992a). In smooth vessel muscle cells they may play a central role in the regulation of muscle toning (DAVIES et al., 1991).

K_{ATP} channels show characteristic pharmacological features. Their opening probability is reduced by hypoglycemic sulfonylureas (e.g. glibenclamide) and increased by K^+ channel openers (KCOs; e.g. pinacidil) (for example see DÖRSCHNER et al., 1999; GROSS et al., 1999 and UHDE et al., 1999).

1.1.1. K_{ATP} channels in pancreatic B-cells

In the resting B-cell (i.e. in the presence of 3 mM of glucose; 37°C) only 6-8% of all K_{ATP} channels are open (PANTEN et al., 1990; SCHWANSTECHER et al., 1992a). The threshold potential for activation of voltage-dependent Ca^{2+} channels is reached when less than 3% of channels are open (COOK et al., 1988; PANTEN et al., 1990; SCHWANSTECHER et al., 1992a). This degree of channel inhibition is induced by glucose concentrations of more than 6 mM. In case of glucose concentrations of 6 mM, the insulin-releasing potencies of sulfonylureas lie within the range of therapeutic plasma concentrations of the free substances and correlate well with the potencies for K_{ATP} channel inhibition (PANTEN et al., 1989). The substrate-induced inhibition of K_{ATP} channels results from a decrease in the ADP concentration and a simultaneous increase in the ATP concentration at the cytosolic side of the plasma membrane (ASHCROFT and RORSMAN, 1991).

1.1.2. K_{ATP} channels in neurons

K_{ATP} channels with the pharmacological properties of B-cell channels have been identified in many different neurons: in demyelinised fibres of the sciatic nerve and in the somata of neurons in hippocampus, neocortex, substantia nigra pars reticulata and caudate nucleus (JONAS et al., 1991; POLITI and ROGAWSKI, 1991; OHNO-SHOSAKU and YAMAMOTO, 1992; SCHWANSTECHER and PANTEN, 1994). Under certain conditions, these channels are probably involved in the regulation of neuronal activity. Thus, their activation in case of hypoxia or ischaemia presumably leads to a hyperpolarisation of the cells and consequently to an inhibition of energy consumption and a delay in cell death.

Neuronal K_{ATP} channels can be activated by KCOs (SCHMID-ANTOMARCHI et al., 1990). Sulfonylureas seem to antagonise the protective effect of K_{ATP} channel activation and thereby intensify tissue damage (JIANG et al., 1992; OHNO-SHOSAKU and YAMAMOTO, 1992; HEURTEAUX et al., 1993). However, therapeutically administered sulfonylureas are unlikely to pass the intact blood-brain barrier (KELLNER et al., 1969; KOLB et al., 1974; SUGITA et al., 1982). These substances possibly reach the central nervous system under ischaemic conditions since prolonged cerebral ischaemia is accompanied by a disturbance of the blood-brain barrier (KUROIWA et al., 1988; GUMERLOCK, 1989).

1.1.3. K_{ATP} channels in heart and skeletal muscle

K_{ATP} channels were detected for the first time in cardiac myocytes (NOMA, 1983). High cytosolic ATP and low cytosolic ADP concentrations may be responsible for the closure of K_{ATP} channels in cardiac muscle under physiological conditions (NICHOLS and LEDERER, 1991). Deficiency of phosphocreatin and consequantively a decrease in cytosolic ATP concentration during hypoxia or ischaemia may lead, however, to opening of the channels. As a result, shortening in action potential duration, loss of cellular K^+ and accumulation of extracellular K^+ resulting in arrhythmia, were observed (GROSS and AUCHAMPACH, 1992b; COETZEE, 1992; WILDE and JANSE, 1994). On the other hand, a protective effect results from shortening of action potential duration since a reduction of Ca^{2+} influx reduces contractility and thus energy consumption. Indeed evidence is presented that the opening of cardiac K_{ATP} channels through KCOs such as nicorandil or cromakalim improves oxygen supply, reduces oxygen consumption in ischaemic areas and has a cardioprotective effect (GROVER, 1994; AUCHAMPACH et al., 1992 and 1994). Sulfonylureas may, on the other hand, intensify tissue damage through inhibition of cardiac K_{ATP} channels in case of cardiac ischaemia (LYNCH et al., 1992).

As in neurons and cardiac muscle the K_{ATP} channel in skeletal muscle cells seems to be closed unless its opening probability is increased by hormones, drugs or a decrease in energy reserves (DAVIES et al., 1991; LIGHT et al., 1994).

1.1.4. K_{ATP} channels in smooth muscle

K_{ATP} channels have also been identified in smooth muscle cells of portal vein. The biophysical properties and the ATP sensitivity of these channels resemble those of the channel in pancreatic B-cells, (SPRUCE et al., 1985, 1987; KAJIOKA et al., 1991; ASHCROFT and ASHCROFT, 1990; DAVIES et al., 1991).

The sensitivity of these channels to sulfonylureas however, is lower than that of the B-cell channel (KOVACS and NELSON, 1991; BEECH et al., 1993; XU and LEE, 1994). In smooth vessel muscles, K_{ATP} channels could be of central importance for the regulation of muscle toning (DAVIES et al., 1991). It is still unclear whether the channels in smooth muscle cells are open under physiological conditions.

KCOs relax vascular and non-vascular smooth muscles by opening K_{ATP} channels (HAMILTON, 1986; COOK and QUAST, 1990; BRAY and QUAST, 1992). Due to their vasodilating properties, these substances lower the blood pressure (EDWARDS and WESTON, 1990; ROBERTSON and STEINBERG, 1990; ANDERSSON and WELSH, 1992).

1.1.5. Structure of K_{ATP} channels

K_{ATP} channels reconstitute a new class of ion channels. They are assembled with tetradimeric stoichiometry (SUR/ $K_{IR}6.x)_4$ (Fig.1) from a regulatory sulfonylurea receptor (SUR1 or SUR2) and a pore-forming inwardly rectifying potassium channel ($K_{IR}6.1$ or $K_{IR}6.2$) (AGUILAR-BRYAN et al., 1995; INAGAKI et al., 1995 and 1996; ISOMOTO et al., 1996; CLEMENT et al., 1997; YAMADA et al., 1997). SUR1/$K_{IR}6.2$ reconstitute the neuronal/pancreatic B-cell K_{ATP} channels (INAGAKI et al., 1995), SUR2A/$K_{IR}6.2$ the cardiac (INAGAKI et al., 1996; OKUYAMA et al., 1998) and SUR2B/$K_{IR}6.1$ (or $K_{IR}6.2$) the vascular smooth muscle-type K_{ATP} channels (ISOMOTO et al., 1996; YAMADA et al., 1997; SCHWANSTECHER et al., 1998).

The sole expression of $K_{IR}6.1$ or $K_{IR}6.2$ does not lead to operative channels (CLEMENT et al., 1997; GRIBBLE et al., 1997). Since point mutations in $K_{IR}6.2$ modulate the conductance of SUR1/ $K_{IR}6.2$ channels in a characteristic manner (SHYNG et al., 1997), it was concluded that $K_{IR}6.2$ or $K_{IR}6.1$, respectively, represents the pore forming subunit of the channel. This

conclusion is supported by the fact that carboxyterminal truncation of $K_{IR}6.2$ results in formation of a potassium conductance in the absence of SUR (TUCKER et al., 1997).

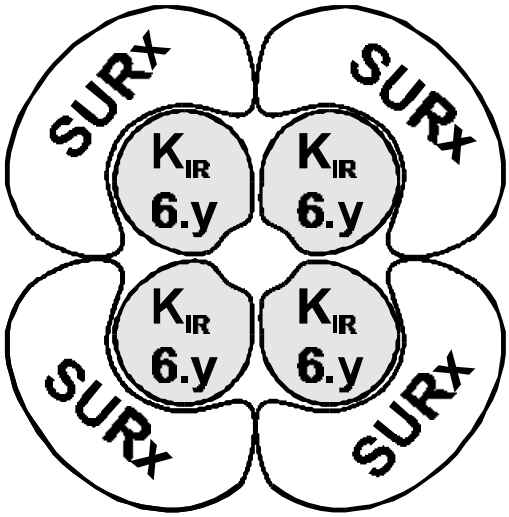


Fig. 1 Tetradimeric structure of ATP-sensitive potassium channels (CLEMENT et al., 1997). The channels consist of four SURx and four $K_{IR}6.y$ subunits.

1.2. Sulfonylurea receptors

A high-affinity binding site for sulfonylureas was detected in membranes from insulin-secreting cell lines and pancreatic islets (GEISEN et al., 1985; PANTEN et al., 1992; GOPALKRISHNAN et al., 1993). The good correlation between the K_{ATP} channel-blocking and insulin-releasing potency of sulfonylureas on the one hand, and the affinity of these drugs for binding to membranes, on the other, suggested that the high-affinity binding site in membrane preparations represents the sulfonylurea receptor (SCHMID-ANTOMARCHI et al., 1987; GAINES et al., 1988; PANTEN et al., 1989; SCHWANSTECHER, 1994). High-affinity binding sites for sulfonylureas were also observed in membranes from brain (KAUBISCH et al., 1982; ASHCROFT and ASHCROFT, 1992; GOPALKRISHNAN et al., 1993), heart muscle cells (FOSSET et al., 1988), smooth muscle cells (KOVACS and NELSON, 1991; GOPALKRISHNAN et al., 1993; ZINI et al., 1991) and skeletal muscle cells (GOPALKRISHNAN et al., 1993).

1.2.1. Sulfonylurea receptors are ABC- proteins

Sulfonylurea receptors (SURs) are members of the family of ABC-proteins (AGUILAR-BRYAN et al., 1995; INAGAKI et al., 1996; ISOMOTO et al., 1996). This family probably is the largest group of proteins for selective transport of substances through biomembranes. The members of this family have a common organisation with four core domains, two transmembrane domains and two nucleotide-binding domains, NBF1 and NBF2, (HIGGINS, 1995). The organisation of an operative transporter is based on two of these basic subunits, i.e. on both NBFs and two transmembrane domains. In most of the hitherto known procaryotic import systems, these subunits seem to be expressed as separate polypeptides and are assembled as hetero- or homodimers into a membrane-bound complex. All bacterial transporters that mediate solute uptake additionaly require a substrate-binding protein (SBP) located outside the cytoplasmic membrane. The individual domains may however be fused (partially or completely) into a single polypeptide and may contain additional regulatory proteins or transmembrane domains (Fig. 2).

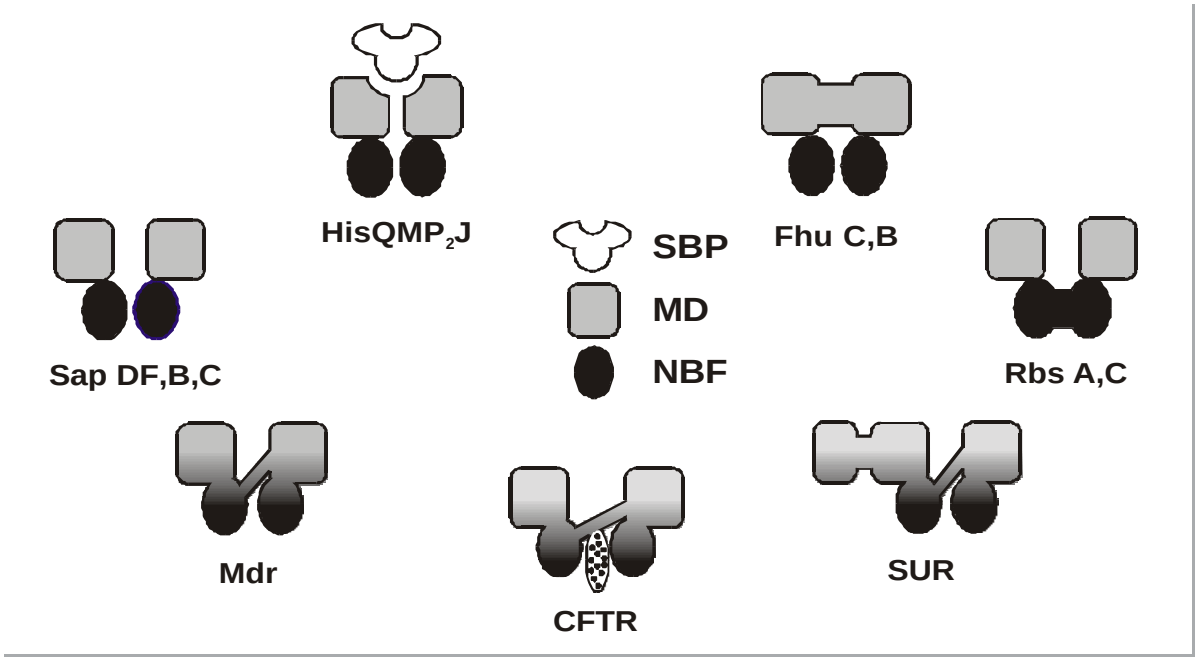


Fig. 2 ***Domain organization of ABC transporters***. A typical ABC transporter consists of four domains, two highly hydrophobic membrane spanning domains (MD, grey colour) which form the translocation pathway, and two nucleotide binding folds (NBFs, black colour) which couple ATP hydrolysis to the transport process. Certain transporters have additional domains (dotted) that are not part of the core transmembrane translocation mechanism (e.g, CFTR). The domains are often encoded as separate polypeptides, however, they may also be fused together in one or several alternative combinations; CFTR = cystic fibrosis transmembrane conductance regulator, $HisQMP_2J$ = histidine permease transporter, Mdr = multi drug resistance protein, Sap DF = (K^+) oligopeptide transporter, Rbs = ribose transpoter, SUR = sulfonylurea receptor and Fhu C = Fe-ferrichrome transporter. For further details see text.

The NBFs contain the characteristic Walker-motifs (WALKER et al.,1982; SARASTE et al., 1990) as well as an additional highly conserved sequence, the so-called linker region (SHYAMALA et al., 1991; MIMURA et al., 1991). The Walker A-motif (GX4GKS/T) forms part of the nucleotide-binding pocket with the highly-conserved lysine (K) residue interacting with the β- and γ- phosphates of ATP (SARASTE et al., 1990; HIGGINS et al., 1992). The Walker B-motif (R/KX6-8LHyd4D; Hyd = hydrophobic amino acid) is thought to be involved in the binding of the adenine ring (BEAUDET and GROS, 1995; FRY et al., 1986), while the linker region (LSGGX3RHydXHydA) probably transduces conformational changes resulting from ATP hydrolysis to other parts of the protein (SHYAMALA et al., 1991; MIMURA et al., 1991).

A comparison of sequences shows that SUR possesses strong homology to the "multidrug-related protein" (MRP), the "P-glycoprotein-related" protein of Leishmania and the "Cystic Fibrosis transmembrane conductance regulator" (CFTR) (AGUILAR-BRYAN et al., 1995; BRYAN and AGUILAR-BRYAN, 1997). AGUILAR-BRYAN et al. (1995) proposed a transmembrane topology for SUR1 consisting of nine transmembrane segments before NBF1 and a further four localised between NBF1 and NBF2 at the carboxy-terminal end. In a more recent study TUSNADY et al. (1997) suggested that SUR1 is formed from eleven transmembrane segments before NBF1 and six between NBF1 and the C-terminally localised NBF2 (Fig. 3).

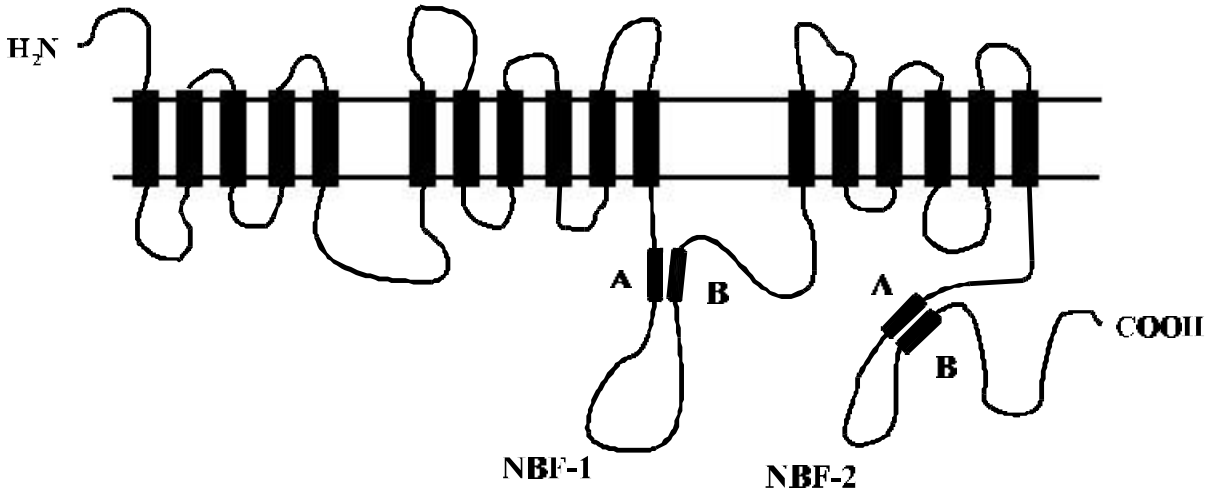


Fig. 3 Transmembrane topology of SUR1. Model proposed by TUSNADY et al. (1997). Both NBFs contain Walker A and Walker B motifs (A,B)

An important recent advance in understanding ABC transporters came when the three-dimensional crystal structure of the periplasmic histidine permease was solved (HUNG et al.,

1998). This protein which is one of the most widely studied ABC transporters in microorganisms imports histidine. The complex is composed of a soluble receptor, the periplasmic histidine-binding protein, HisJ, and a four-subunit membrane-bound complex ($HisQMP_2$) composed of HisQ and HisM (the two hydrophobic subunits) and of two identical HisP subunits (P_2) that carry the highly conserved nucleotide-binding domain (DOIGE et al., 1993) (Fig. 2 and Fig. 4). The membrane-bound complex of the histidine permease has been purified and reconstituted into proteoliposomes (PLS), and shown to hydrolyse ATP and translocate histidine in a manner strictly dependent on the presence of HisJ (LIU et al , 1997; see Fig.2). In the absence of the binding protein, the complex hydrolyses ATP at a low rate only (LIU et al., 1997).

The recent resolution of the three-dimensional structure of HisP at 1.5 Å clearly shows the presence of an ATP-binding pocket containing an ATP molecule (HUNG et al., 1998). The structure provides a model for the binding of ATP to an NBF and thus a basis for understanding properties of ABC transporters such as SUR1.

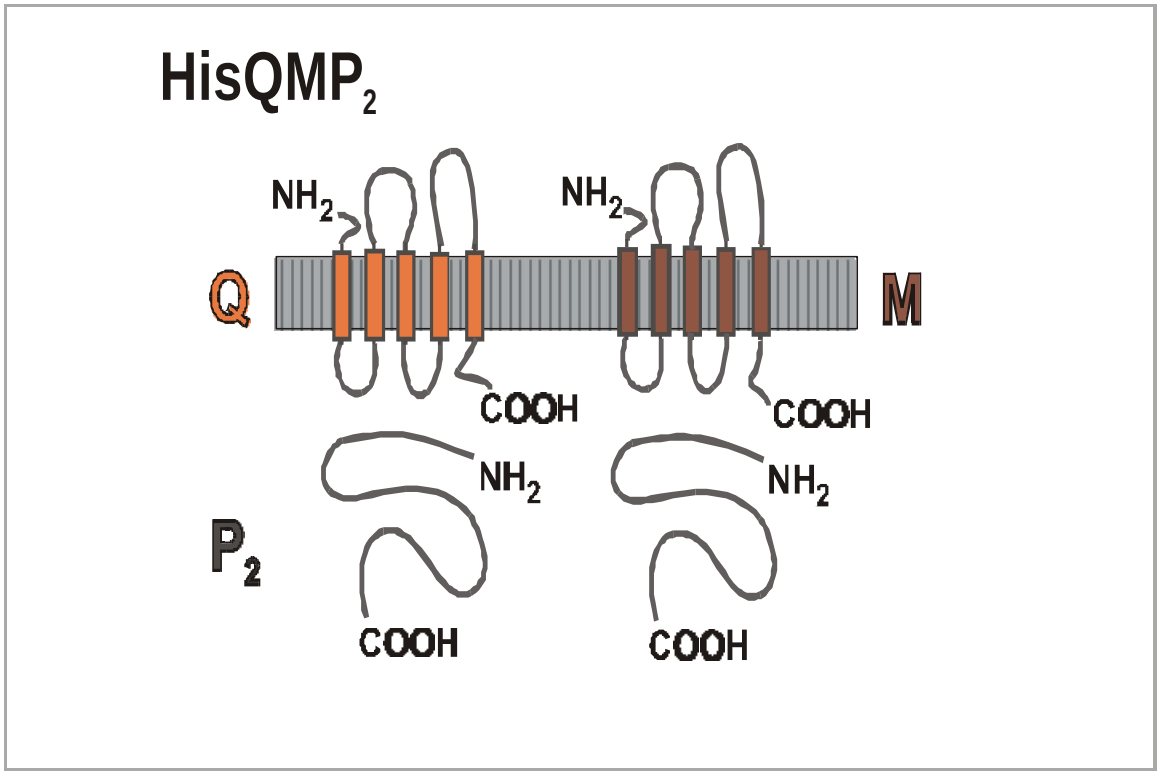


Fig. 4 Transmembrane topology of $HisQMP_2$. For details see text.

1.2.2. Cloning of sulfonylurea receptors

In 1995 AGUILAR-BRYAN et al. succeeded in purifying the high-affinity sulfonylurea receptor (SUR1) from HIT T15 cells (a hamster B-cell line). After microsequencing of the amino-terminus, the receptor was cloned from cDNA libraries of HIT T15 cells and RINm5F cells (a rat insulinoma cell line). In 1996, two iso-forms of the sulfonylurea receptor were cloned: SUR2A from rat brain and SUR2B from mouse heart (CHUTKOW et al., 1996; INAGAKI et al., 1996; ISOMOTO et al., 1996). SUR2A and SUR2B are splice products of a single gene, differing only in their C-termminal 42-45 amino acids (ISOMOTO et al., 1996). While the RNA for SUR2A was observed mainly in heart and skeletal muscle, the RNA for SUR2B could be detected in all examined tissues (INAGAKI et al., 1996 ISOMOTO et al., 1996).

Due to the distinct pharmacology and tissue distribution of the two iso-forms, it is assumed that SUR2A represents the sulfonylurea receptor of heart and skeletal muscle and SUR2B that of the vascular smooth muscle (ISOMOTO et al., 1996).

1.2.3. Mutations in SUR1 and K_{IR}6.2 cause PHHI

Defects in four genes (Glucokinase, glutamate dehydrogenase, SUR1 and K_{IR} 6.2) are known to cause familial persistent hypoglycemia of infancy, PHHI (THOMAS et al., 1995, 1996a and b; AGUILAR-BRYAN and BRYAN,1996; DUNNE et al., 1997). Mutations in SUR1, however, are the most common cause (NESTOROWICZ et al., 1998) and up to now more than 40 are known. All seem to be recessive. However, detailed studies on heterozygotes have not yet been done, so it cannot be excluded that the heterozygous state is associated with a slight disturbance in insulin secretion.

1.2.3.1. PHHI results as a consequence of reduced open probability of SUR1/K_{IR} 6.2 channels

On the basis of their functional effects, mutations in SUR1 may be grouped into two classes. Class I mutations result in the total loss of functional K_{ATP}-channel activity (e.g. F1388), while class II mutations impair the ability of MgADP to enhance K_{ATP}-channel activity (e.g. G1479R). The latter mutations abolish the ability of the channel to respond to changes in metabolism and cause it to be permanently closed in the intact B-cell.

Both classes of PHHI mutations result in a lack of K_{ATP}-channel activity in the B-cell, even at low blood glucose concentrations. This results in a continuous depolarisation of the B-cell and a high resting intracellular Ca^{2+} concentration, which explains the constitutive insulin secretion characteristic of PHHI patients (for a review see ASHCROFT and GRIBBLE, 1999).

1.2.3.2. Incidence and clinical presentation of PHHI

The disease which is characterised clinically by excessively elevated circulating insulin concentrations despite severe hypoglycemia (STANLEY and BAKER, 1976; AYNSLEY-GREEN, 1981; LANDAU et al., 1982) is most frequently diagnosed in the newborn or in infants, although a number of cases have been reported in adults as well (HARNESS et al., 1981). In a non-inbred Caucasian population it is estimated to occur at a frequency of 1 in 50,000 live births (BRUINING, 1990). However, reported incidence is 1 per 2.675 live births in an arabic population with a high degree of consanguinity (MATTHEW, et al., 1988). An approximate calculation using the Hardy-Weinberg formula suggests that the frequency of the heterozygous genotype in the general population is ~0.9%, or ~450 times that of the recessive homozygote.

The clinical presentation of PHHI is variable (LANDAU and SCHILLER, 1991). Some patients develop severe hypoglycemia minutes to hours after birth while others do not have severe symptoms for several weeks or months. The clinical symptoms of both the severe and the milder forms of the disease have been noted to diminish with age (STANLEY and BAKER, 1976; GRANT et al., 1986; LANDAU and SCHILLER, 1991).

1.3. Modulation of K_{ATP} channel activity

1.3.1. Cytosolic nucleotides

K_{ATP}-channels are inhibited by intracellular ATP in the presence or absence of Mg (IC_{50} = 10µM; COOK and HALES, 1984). This effect of ATP does not require hydrolysis of the nucleotide as it can not be imitated by non hydrolysable ATP-analogues like AMP-PNP or AMP-PCP. In addition to ATP, free ADP, diadenosinopolyphosphate, free GTP and GDP reduce K_{ATP}-channel activity (COOK and HALES, 1984; MARTIN et al., 1998, RIBALET and CIANI, 1987). The binding site for these inhibitory nucleotides is localised on $K_{IR}6.2$, most probably within the intracellular proximal C-terminal region (see Fig. 1) (TUCKER et al., 1997, 1998; DRAIN et al., 1998; MARKWORTH et al., 2000). The Mg-complexes of cytosolic nucleotides like ADP and GDP stimulate K_{ATP} channel activity in the presence as well as in the absence of inhibitory ATP (DUNNE and PETERSEN, 1986a and b; FINDLAY, 1987).

A rise in the ratio of cytosolic concentrations of ATP and ADP thus leads to an inhibition of K_{ATP} channel activity and consequently to depolarisation of the plasma membrane, opening of voltage-dependent Ca^{2+} channels and an increase in Ca^{2+} influx . The resultant rise in cytosolic Ca^{2+} concentration induces the release of insulin (Fig.5).

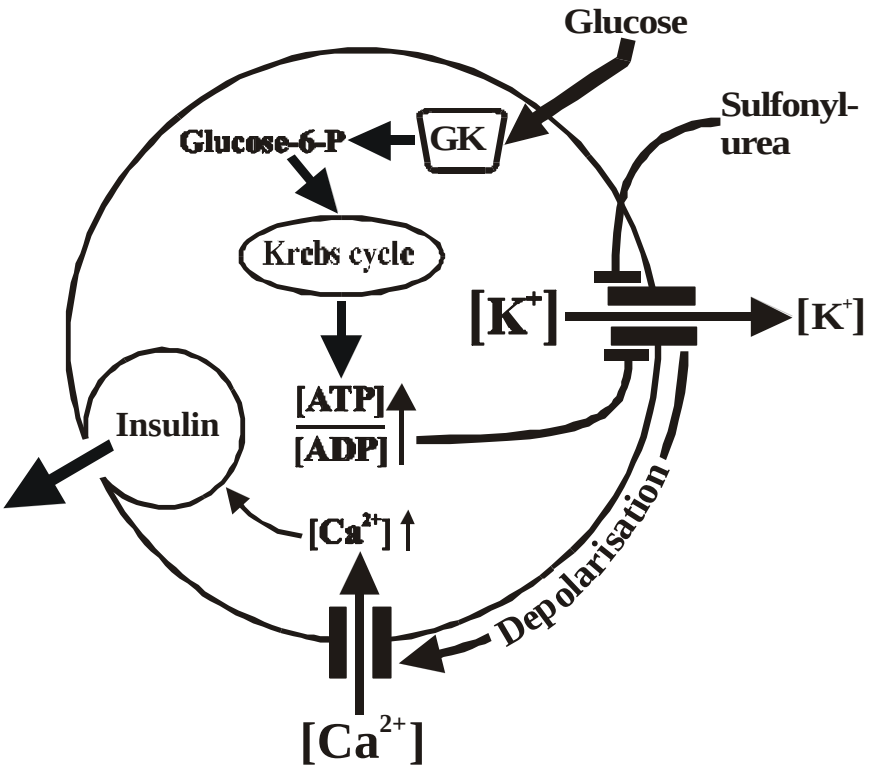


Fig. 5 Stimulation of insulin secretion in the B-cell (SCHWANSTECHER, 1994). Inhibition of the opening activity of K_{ATP} channels by sulfonylureas or a rise in the ratio of cytosolic concentrations of ATP and ADP ([ATP]/[ADP]), leads to depolarisation of the plasma membrane, opening of voltage-dependent Ca^{2+} channels and an increase in Ca^{2+} influx . The resultant rise in cytosolic Ca^{2+} concentration induces the release of insulin. An increase in the [ATP]/[ADP] ratio occurs, if the mitochondrial citrate cycle is sufficiently activated by nutrients (e.g. glucose). A rise in the extracellular glucose concentration increases the phosphorylation of glucose through glucokinase (GK), the rate-limiting enzyme for the metabolism of glucose in the B-cell.

1.3.2. Sulfonylureas

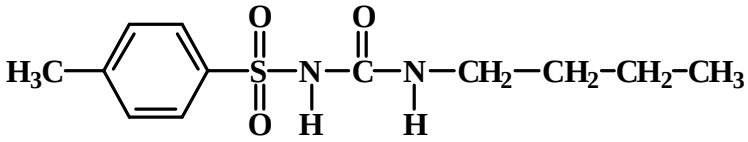
1.3.2.1. Structure

All sulfonylurea drugs used for lowering the blood glucose concentration have common structural features, a benzene-sulphonic acid and a urea group (Fig. 6; PANTEN et al., 1992) Tolbutamide is regarded as a typical representative of the so-called first generation sulfonylurea drugs. Up to the introduction of glibenclamide (B in Fig. 6), it was the most frequently used drug in the therapy of type II diabetes mellitus. Glibenclamide was the first commercially available second generation sulfonylurea (AUMÜLLER et al., 1966) and is the most prescribed oral antidiabetic drug in the world. While the first generation sulfonylurea drugs (e.g. tolbutamide) have to be administered in gram/day doses, the second generation drugs (e.g. glibenclamide and glipizide) are given in milligram/day doses. An additional

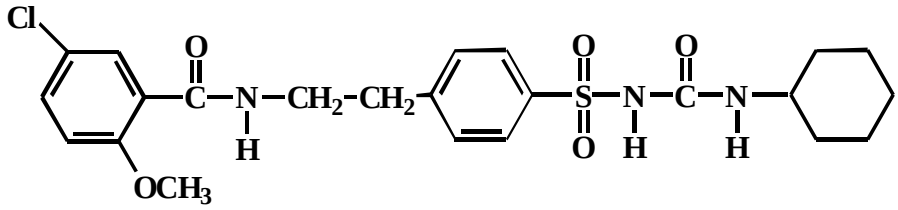
carboxamido group and its substituents lead to an increased effectiveness of these molecules (Fig. 6).

Sulfonylurea compounds are weak organic acids which are mostly anionic at physiological pH. However, their non-dissociated forms are very lipophilic (PANTEN et al., 1989).

A



B



C

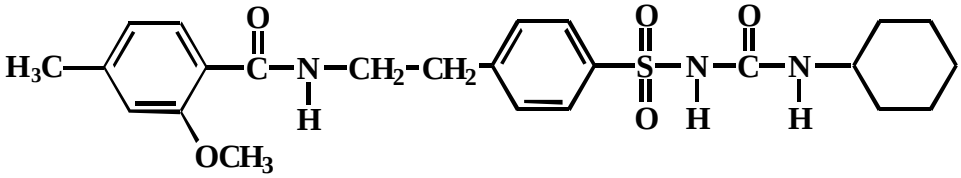


Fig. 6 Structural formula of tolbutamide (A), glibenclamide (B), glipizide (C).

1.3.2.2. Mechanism of action

Sulfonylureas stimulate the secretion of insulin from pancreatic B-cells. This effect is independent of the presence of nutrients (e.g. glucose) or other stimuli of insulin secretion (MALAISSE et al., 1967; GORUS et al., 1988). It is based on the fact that these substances reduce the K^+ permeability and thus depolarise the B-cell membrane (Fig. 5; DEAN and MATTHEWS, 1968; MATTHEWS, 1985; HENQUIN, 1987). The depolarisation of the plasma membrane leads to an opening of voltage-dependent Ca^{2+} channels and an increase in Ca^{2+} influx into the cytosol. The resultant rise in the cytosolic Ca^{2+} concentration induces the release of insulin (HENQUIN, 1987).

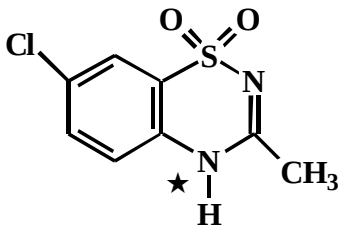
Patch-clamp experiments show that the sulfonylurea-induced decrease of K^+ permeability is due to an inhibition of the opening activity of the K_{ATP} channel in the B-cell membrane (Fig. 5).

1.3.3. Potassium channel openers

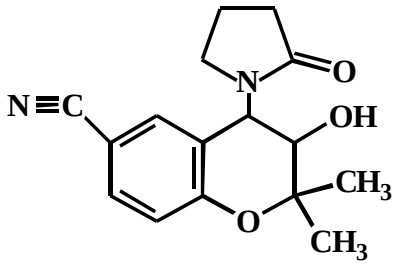
1.3.3.1. Introduction

KCOs comprise a structurally diverse group of substances which activate K_{ATP} channels. These drugs [e.g. benzopyranes such as cromakalim (D in Fig. 7) or levcromakalim, nicotinamides such as nicorandil (E in Fig. 7), pyrimidine derivatives such as minoxidilsulfate (F in Fig. 7), cyanoguanidines such as pinacidil (B in Fig. 7) or P1075 (C in Fig. 7) and benzothiadiazines such as diazoxide (A in Fig. 7)] increase the outflow of $^{86}Rb^+$ or $^{42}K^+$ in efflux experiments with vascular smooth muscle (COOK and QUAST, 1990), resulting in hyperpolarisation, a reduced activity of voltage-dependent Ca^{2+} channels and a reduction of electrical activity. The resultant decrease in the cytosolic Ca^{2+} concentration induces, for example, an inhibition in the activity of secretory cells or a relaxation of muscle cells (EDWARDS and WESTON, 1995)

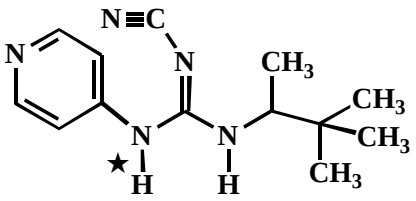
A



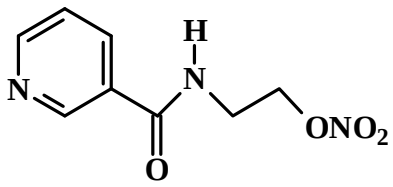
D



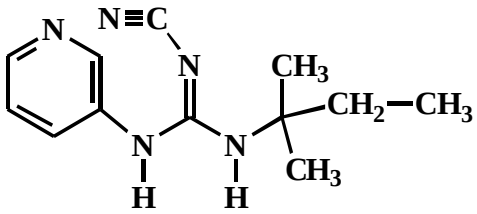
B



E



C



F

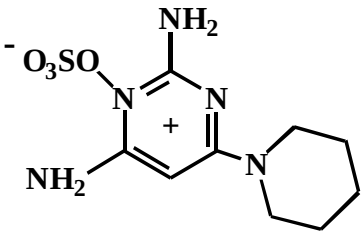


Fig. 7 Chemical structures of diazoxide (A), pinacidil (B), P1075 (C), cromakalim (D), nicorandil (E) and minoxidilsulfate (F). In case of diazoxide and pinacidil, the forms prevailing in the tautomeric balance are shown (WOHL, 1970; PETERSEN et al., 1978). The dissociating protons are indicated by ★ (pK_a = 8.6 for diazoxide; pK_a = 10.8 for pinacidil)

1.3.3.1.1. Diazoxide

Diazoxide (A in Fig. 7) a substance related to sulfonylureas, inhibits insulin secretion in pancreatic B-cells of (SELZER and ALLEN, 1965; PORTE, 1968). Moreover, it induces hyperglycemia (DOLLERY et al., 1962; TALBACHNIK and GULBENKIAN, 1968) and causes relaxation of smooth vascular muscles in concentrations similar to those required for the effect on pancreatic B-cells (GILMAN et al., 1985; QUAST and COOK, 1989).

The observation that diazoxide induces a rise of ^{86}Rb-efflux in B-cells provided first clues to the mechanism of action of this substance (HENQUIN and MEISSNER, 1982). Increased ^{86}Rb-efflux coincided with hyperpolarisation of the cells and suppression of normal electrical activity. HENQUIN and MEISSNER (1982) concluded that inhibition of insulin secretion from B-cells by diazoxide is due to an increase in K^+ permeability of the cell membrane. A few years later patch-clamp studies revealed that diazoxide acts through the same K_{ATP} channel that is closed by sulfonylureas (Fig. 6; TRUBE et al., 1986). In cell-attached experiments, diazoxide antagonised both nutrient- and tolbutamide-induced channel inhibition.

In cultured neurones and brain slices, it was shown that diazoxide increases the opening probability of the neuronal K_{ATP} channel as well (OHNO-SHOSAKU and YAMAMOTO, 1992; SCHWANSTECHER and PANTEN, 1994). Activation of the K_{ATP} channel of smooth vascular and cardiac muscle cells was also observed (BEECH et al., 1993; KAJIOKA et al., 1991; NELSON and QUAYLE, 1995; D'HAHAN et al., 1999).

1.3.3.1.2. Pinacidil

Pinacidil (B in Fig. 7) also belongs to the group of KCOs (BRAY et al., 1987; COOK et al., 1988). This substance is a racemate, with the (-)-enantimer possessing a considerably stronger potassium channel-opening effect (COOK et al., 1988). In addition to pinacidil, more potent KCOs have been synthesized, such as P1060 (IBBOTSON et al., 1993) and P1075 (C in Fig. 7; BRAY and QUAST, 1992).

In smooth muscle cells, very low concentrations of pinacidil have been shown to activate K^+-outflow (COOK and QUAST, 1990; QUAST, 1992). The substance can open K_{ATP} channels in B-cells, neurons and in heart as well (GARRINO and HENQUIN, 1987; SCHWANSTECHER et al., 1992c; SCHWANSTECHER 1994; SCHWANSTECHER and BASSEN, 1997; ESCANDE et al., 1988 and 1989). Specific binding of [^{3}H]P1075 (C in Fig. 7) has been detected in intact aortic-rings of the rat (BRAY and QUAST, 1992).

1.3.3.1.3. Other potassium channel openers

Of all KCOs only diazoxide and pinacidil induce an increase in the opening probability of K_{ATP} channels in B-cells. Nicorandil (E in Fig. 7) is ineffective whilst minoxidil sulphate (F in Fig. 7) and cromakalim (D in Fig. 7) induce channel inhibition (PLANT et al., 1989). For all these substances, the effects on insulin secretion correspond well to their effects on the K_{ATP} channel.

The K_{ATP} channel in cardiac muscle cells is not only activated by pinacidil and diazoxide but also by cromakalim, RP49356 and nicorandil (ESCANDE et al., 1988 and 1989; THURINGER and ESCANDE, 1989; SHEN et al., 1991). In addition to pinacidil and diazoxide, a series of other KCOs are effective on the smooth muscle, e.g. levcromakalim, nicorandil and minoxidilsulphate (WESTON and EDWARDS, 1992).

1.3.3.2. Therapeutic potential

The known KCOs (with the exception of diazoxide) show a relative specificity for vascular and non-vascular smooth muscle (QUAST, 1996).

These substances effectively lower blood pressure and could therefore be useful in the treatment of hypertension and other cardiovascular diseases (e.g. angina pectoris) (EDWARDS and WESTON, 1990; QUAST, 1992; LAWSON, 1996a and b). In concentrations lowering blood pressure KCOs have little effect on the central nervous system. This is most probably due to poor penetration of the substances through the blood-brain barrier. In addition, the substances have no effect on plasma insulin levels at therapeutic concentrations (QUAST and COOK, 1989). Diazoxide is an exception in this case, as it lowers blood pressure and inhibits insulin secretion (GILMAN et al., 1985).

Levcromakalim (unlike verapamil or nitroprusside sodium) selectively improves collateral blood perfusion in isolated rabbit ear after arterial occlusion (RANDALL and GRIFFITH, 1992). In vivo, a series of KCOs (levcromakalim, aprikalim, nicorandil and pinacidil) protects the myocardium against the residual effects of transient ischaemia (GROSS, 1991; GROVER, 1991; ESCANDE and CAVERO, 1992; GROSS and AUCHAMPACH, 1992a; AUCHAMPACH et al., 1994). Cardioprotection is abolished by glibenclamide (GROSS and AUCHAMPACH, 1992b).

KCOs could also be suitable for treating further illnesses. In several animal models of epilepsy, it was shown that these substances reduce or prevent the activity of seizures (GANDOLFO et al 1989). In clinical studies, cromakalim (0.5 mg per day) lead to an improvement of symptoms in asthma patients (WILLIAMS et al., 1990). However, clinical

application of KCOs is hampered by its insufficient tissue selectivity. Therefore, development of highly efficient drugs against diseases like asthma, hypertension, ischaemia, arrhythmia and epilepsy will most probably require development of tissue-selective compounds.

1.3.3.3. Identification of the receptor binding sites for KCOs

In membranes from B-cells, insulin-secreting tumour cells and cerebral cortex, diazoxide and pinacidil have been shown to inhibit high-affinity binding of [^{3}H]glibenclamide (SCHWANSTECHER and RIETZE, 1990; SCHWANSTECHER et al., 1990, 1991a and b, 1992a, b, c and d). These effects required the presence of MgATP and were not observed in the absence of Mg^{2+} or presence of non-hydrolysable ATP- analogues. The findings indicated that binding of KCOs to their receptor site requires hydrolysis of ATP and that the binding sites for KCOs and sulfonylureas are coupled in a negative allosteric manner. SCHWANSTECHER et al. (1998) have shown that the receptor binding site for KCOs is localised on SURs and that a conformational change induced by ATP binding and hydrolysis in both NBFs presumably results in a strong increase in KCO affinity and thus is necessary for binding of KCOs (see also UHDE et al., 1999). Non-hydrolysable ATP analogues, such as AMP-PNP and AMP-PCP as well as ADP in the presence of an ATP capturing system were not able to induce KCO binding. For the conformational change divalent cations are required and have a rank-order effectiveness (Mg = Mn > Zn > Ca > Sr) characteristic for ATPase-activity of other ABC-proteins (SCHWANSTECHER et al., 1998).

2. Aims of the study

The aims of the study were:

(1) To analyze the effect of PHHI-inducing point mutations on diazoxide and glibenclamide binding to SUR1.

(2) To establish and test a structural model for the NBFs of SUR1 based on homology modeling.

3. Materials and methods

3.1. Cultivation, passaging and preservation of COS7-cells

3.1.1. Solutions and media for cell culture

A) Penicillin/streptomycin stock solution (20000 IU/ml and/or 20000 µg /ml)

1.2 g of penicillin G-sodium (Nr. 321-42, Biochrom) and 2.0 g streptomycin sulfate (Nr. 331-26, Biochrom) were dissolved in 100 ml 0.9% sodium chloride solution and stored at -20°C.

B) Dulbecco´s Modified Eagle´s medium (DMEM) + 10% FCS

13.5 g DMEM (powder; Nr. T 043-10, Biochrom) and 2.2 g of sodium hydrogencarbonate (Nr. 6329, Merck; final concentration 26 mM) were dissolved in approx. 800 ml bidistilled water. 5 ml of the penicillin/streptomycin stock solution (final concentration 100 IU/ml and/or 100 µg/ml) were added and the solution was adjusted to pH 7.15 with 1N HCl or 1N NaOH, respectively. The volume was filled up to 1000 ml with bidist. water. The solution was sterile-filtered using a stainless steel vacuum filtration apparatus (Nr. SM 16268 107500, Sartorius) with preliminary filter (Nr. 13400-130 K, Sartorius) and main filter (0.2 µm; Nr. 11307-142, Sartorius). The medium was stored at 4°C for up to 8 weeks. Immediately before use, 100 ml of foetal calf serum (FCS; sterile-filtered, Kraeber Ltd. & Co; and heat-inactivated for 30 mins at 56°C) were added and the resultant medium was kept in a water bath at 37°C.

C) Freezing medium (DMEM + 20% FCS)

Under laminar air flow 3 ml of FCS were added to 12 ml DMEM (+ $NaHCO_3$ and penicillin/streptomycin; 3.1.1. B)

D) DMSO solution (Freezing medium + 20% DMSO)

Under laminar air flow 12 ml freezing medium (see 3.1.1. C) were mixed with 3 ml of sterile DMSO (Nr. 1.02952, Merck).

E) Trypsin/EDTA solution

100 ml of trypsin/EDTA solution (0.5 %/0.2% in 10x PBS without Ca^{2+} and Mg^{2+}; L-2153, Biochrom) were mixed with 900 ml sterile bidist. water, split up into 50 ml aliquots and stored at -20°C.

F) Phosphate buffer (CMF- PBS)

8.0 g sodium chloride (Nr. 1.06404, Merck), 0.2 g potassium chloride (Nr. 1.04936, Merck), 1.15 g anhydrous disodium hydrogen phosphate (Nr. 1.06586, Merck) and 0.2 g potassium dihydrogen phosphate (Nr. 4873, Merck) were dissolved in 900 ml bidist. water. The solution was adjusted to pH 7.3 with 1N NaOH or 1N HCl, respectively and diluted to 1000 ml with bidist. water. The solution was then autoclaved in aliquots.

3.1.2. Cultivation of COS7-cells

The COS7 cell (CV-1, Origin of SV40, GLUZMAN, 1981) is a derivative of a permanent kidney cell line of the African green guenon (CV-1) infected with SV40 viruses (**S**imian **V**irus). The monolayer cells were cultured at 37°C and 5% CO_2 in DMEM with 10% FCS (see 3.1.1. B) and passaged at intervals of 3 - 4 days.

3.1.3. Passaging of COS7-cells

After removing the culture medium from the Petri dishes the cells were carefully rinsed with 5 ml CMF-PBS (see 3.1.1. F). 4 ml of trypsin/EDTA solution (see 3.1.1. E) were added to each of the Petri dishes which were subsequently incubated for two to five minutes at 37°C. Trypsinization was stopped by adding 3 ml of fresh culture medium (DMEM+10% FCS, 3.1.1. B). By gently tapping the Petri dishes the cells still stucking to the ground were released. The cell suspension of each Petri dish was then transferred to a sterile centrifuge tube (Sarstedt) and sedimented by centrifugation (5 min at 100 g and 4°C, Heraeus Megafuge 1.0 R, rotor 2705, 900 rpm.). After removing the supernatant and determining the cell count, each of the cell pellets was resuspended in 10 ml culture medium and split up in a ratio of 1:3 to 1:5 into fresh Petri dishes so that approx. 500000 cells were placed in each dish.

3.1.4. Cryopreservation of COS7-cells

For cryopreservation, the cells from densely grown culture plates were trypsinized as described in 3.1.3. They were subsequently resuspended in 1.5 ml of pre-cooled freezing medium (4°C, see 3.1.1. C) and mixed with 1.5 ml of cold DMSO solution (4°C, see 3.1.1. D). 1.5 ml aliquots of this suspension were pipetted into a sterile pre-cooled cryotube (4°C). The tubes were kept at -20°C for 1 h followed by -70°C for 24 h and then stored in liquid nitrogen (-196°C).

3.2. Preparation of mutations

3.2.1. Solutions for mutagenesis

A) PCR-primer (10 pmol/µl)

10 nmol lyophilized PCR-primer (Roth) were dissolved in 100 µl autoclaved bidist. water. 80 µl of this solution were autoclaved and diluted with 720 µl bidist. water.

B) 0.5 M EDTA (pH 8.0)

93.1g ethylenediaminetetraacetic acid (EDTA; Nr.1.08418, Merck) were dissolved in 350 ml bidist. water, adjusted with NaOH to pH 8.0 (Nr. 106498, Merck). The solution was made up to 500 ml with water and autoclaved.

C) TAE buffer

242 g Tris (4855.2, Roth), 57.1 ml glacial acetic acid (Nr. 5421.3, Bernd Kraft GmbH) and 100 ml 0.5 M EDTA solution were dissolved in bidist. water to a final volume of 1000 ml. 20 ml of this stock solution were diluted with bidist water to 1000ml.

D) Loading buffer

250 mg xylene cyanol (Nr. 1.10590, Merck), 250 mg bromophenol blue (Nr. B-8026, Sigma), 25 g ficoll 400 (Nr. 17-0400-01, Pharmacia) and 10 ml 0.5 M EDTA were dissolved in bidist. water to make 100 ml. The buffer was then autoclaved.

3.2.2. QuikChange site-directed mutagenesis

Mutations were induced by the "QuikChange site-directed mutagenesis" method (see 4.1. for further details). The PCR was carried out in a Biometra Personal Cycler in 0.5 ml Eppendorf safe-lock reaction tubes (Nr. 0030 121.23). Two different oligonucleotide primers (25-45 bases) were used. Each reaction tube contained 1 µl of dNTP mix, 5-50 ng of double stranded DNA (dsDNA) as template, 125 ng of oligonucleotide primer 1, 125 ng of oligonucleotide primer 2, as well as 5 µl DNA polymerase reaction buffer, 10 times concentrated, and 2.5 units Pfu DNA-polymerase (both Nr. 600153, Stratagene) in a total volume of 50 µl. The sets were overlayed with about 100 µl mineral oil (Nr. M 5904, Sigma). Each PCR reaction comprised 12 to 18 reaction cycles. In each cycle, the PCR reaction was heated for 1 min at 95°C to denature the DNA, followed by 1 min at a temperature of about 5°C below the melting temperature of the primer (between 50 and 65°C), which allowed binding to the template DNA. dsDNA could be extended by action of the polymerase. Finally, as the reaction mixture was heated for 2 minutes/Kb of plasmid length to 68°C (which is the optimal working temperature of the polymerase) a complete dsDNA including the vector was obtained (see 4.1.2).

3.2.3. Digestion of the products

1 µl of *Dpn* I restriction enzyme (10 U/µl) was added directly to each amplification reaction and the reaction mixture was then spinned down in a microcentrifuge for 1 min at 13000 rpm. Finally, each reaction was incubated at 37°C for 1 hour to digest the parental (i.e. the non-mutated) supercoiled dsDNA.

3.3. Transformation and plasmid preparation

3.3.1. Solutions

A) Glucose solution (1 M, sterile)

19,82 g of glucose monohydrate (Nr. 8342, Merck) were dissolved in 90 ml bidist. water. The solution was then made up to 100 ml with bidist. water and subsequently sterile-filtered. Filtration was carried out using "Filtropur S 0.2" pore size 0.2 µm, Nr. 83.1826.001, Sarstedt).

B) Potassium chloride stock solution (0.25 M)

1.86 g of potassium chloride (Nr. 4936, Merck) were dissolved in 100 ml bidist. water. The solution was adjusted to pH 7.0 with 1 N KOH and autoclaved.

C) Luria-Bertani medium (LB medium)

10 g Select Peptone 140 (Nr. 30392-021, Gibco), 5 g yeast extract (Nr. 30393-029, Gibco) and 10 g sodium chloride (Nr. 1.06404, Merck) were dissolved in 950 ml bidist. water. The solution was adjusted to pH 7.0 with 5 N NaOH, made up to 1000 ml and subsequently autoclaved.

D) SOB medium

20 g Select Peptone 140 (Nr. 30392-021, Gibco), 5 g yeast extract (Nr. 30393-029, Gibco) and 0.5 g sodium chloride (Nr. 1.06404, Merck) were dissolved in 950 ml bidist. water. Then 10 ml of 0.25 M potassium chloride solution were added (see 3.3.1 B). The solution was adjusted to pH 7.0 with 5 N NaOH filled up to 1000 ml and subsquently autoclaved.

E) SOC-medium

SOB medium (see 3.3.1. D) was cooled to approx. 60°C after being autoclaved. 2 ml of a 1 M sterile glucose solution were added (see 3.3.1. A) to 100 ml SOB medium.

F) Ampicillin stock solution (50 mg/ml)

1 g ampicillin (Nr. 835242, Boehringer Mannheim) was dissolved in 20 ml bidist. water, sterile-filtered and stored at -20°C in the dark. Sterile filtration was carried out as described (3.3.1. A).

G) LB-Agar plates with 100 µg/ml ampicillin

15 g Agar-Agar (Nr. 5210.1, Roth) were added to 1 l LB medium (see 3.3.1. C) and subsequently the medium was autoclaved. When the medium was cooled down to approx. 50°C, 2 ml of ampicillin stock solution (see 3.3.1 F) were added and the plates were casted under laminar air flow (approx. 30 ml per Petri dish of 94 mm diameter). The plates were stored at 4°C up to use.

H) Medium A for production of transformation competent cells

0.493 g of magnesium sulphate (Nr. 5886, Merck) and 0.4 g of glucose monohydrate (Nr. 8342, Merck) were dissolved in 200 ml LB medium (see 3.3.1 C) and the solution was subsequently sterilized as described (3.3.1. A).

I) Medium B for production of transformation competent cells

36 ml glycerin (anhydrous, pure, Nr. 4093, Merck), 12 g of polyethylene glycol 8.000 (Nr. P-2139, Sigma) and 0.296 g of magnesium sulphate (Nr. 5886, Merck) were dissolved in 100 ml LB medium (see 3.3.1 C) and the solution was subsequently sterilized as described (3.3.1.A).

J) Resuspending buffer for plasmid preparation

6.066 g Tris (Nr. 100 849, Paesel + Lorei) and 3.722 g EDTA (Nr. 1.08418, Merck) were dissolved in 800 ml sterile bidist. water, The pH-value was adjusted to 8.0 with 1 N HCl and the solution was filled up to 1000 ml with sterile bidist. water. In 100 ml of this solution 10 mg RNAse A (Cat. No. 19101, Qiagen) were dissolved. The resuspending buffer could be stored at 4°C for up to 6 months.

K) Lysis buffer for plasmid preparation

8 g of NaOH (Nr. 6462, Merck) and 10 g sodium dodecylsulfate (special quality, Nr 1028693, Boehringer Mannheim) were dissolved in 1000 ml sterile bidist. water.

L) Neutralization buffer for plasmid preparation

294.42 g of potassium acetate (for molecular biology, no. 1.04830, Merck) were dissolved in 500 ml sterile bidest. water, the pH-value was adjusted with glacial acetic acid to 5.5 and completed with sterile bidest. water to 1000 ml.

M) Equilibrium buffer for plasmid preparation

43.83 g of sodium chloride (no. 1.06404, Merck) and 10.46 g 4-morpholinepropanesulfonic acid (MOPS; Nr. 6979.2, Roth) were dissolved in 700 ml sterile bidist. water and the pH-value was adjusted to 7.0 with 1 N NaOH. After addition of 156.25 ml of ethanol and 1.5 ml triton X-100 (Nr. 789704, Boehringer Mannheim) the buffer was filled up with sterile bidist. water to 1000 ml.

N) Washing buffer for plasmid preparation

58.44 g of sodium chloride (Nr. 1.06404, Merck) and 20.92 g 4-morpholinepropanesulfonic acid (MOPS; Nr. 6979.2, Roth) were dissolved in 1500 ml sterile bidist. water and the pH-value was adjusted with 1 N NaOH to 7.0. After addition of 312.5 ml of ethanol the solution was filled up with sterile bidist. water to 2000 ml.

O) Elution buffer for plasmid preparation

73.05 g of sodium chloride (Nr. 1.06404, Merck) and 6.066 g Tris (Nr. 100 849, Paesel + Lorei) were dissolved in 800 ml sterile bidist. water and the pH-value was adjusted with 1 N HCl to 8.5. After addition of 156.25 ml of ethanol the solution was filled up with sterile bidist. water to 1000 ml.

P) Tris/EDTA buffer

1.21 g Tris (Nr. 100 849, Paesel + Lorei) and 372.2 mg EDTA (no. 1.08418, Merck) were dissolved in 800 ml bidist. water, the pH-value was adjusted with 1 N HCl to 8.0 and the solution was filled up with bidist. water to 1000 ml. The buffer was split up into aliquots of 100 ml and autoclaved.

3.3.2. Production of transformation competent cells

The production of transformation competent cells was done by use of polyethylene glycol (PEG method). 5 ml LB medium (see 3.3.1 C) were inoculated with E. coli cells (Epicurian Coli XL1 - Blue, Stratagene) and incubated overnight at 37°C in a shaking incubator (Innova 4300, New Brunswick Scientific). The next morning, 50 ml medium A (see 3.3.1. H) were inoculated with 500 µl of the overnight culture and incubated at 37°C in the shaking incubator until an optical density of 0.55 at 578 nm was achieved (spectrophotometer Uvikon 932, Kontron Instruments). Subsequently, the bacterial suspension was transferred to a centrifuge tube and incubated 10 min on ice, then 10 min at 4°C and centrifuged at 1500 g (cool centrifuge GR 2022 with rotor 290.9, Jouan; 2900 rpm). The sediment was resuspended in 500 µl ice-cold medium A (see 3.3.1. H). After addition of 2.5 ml medium B (see 3.3.1 I) the bacterial suspension was mixed carefully and split up into aliquots of 100 µl. The competent cells were frozen at -80°C and stored for up to one year.

3.3.3 Transformation

50 µl competent cells (see 3.3.2.) were pipetted into a sterile reaction tube. After addition of 2 µl of plasmid DNA solution (50 µg/ml) it was shaken carefully and kept for 30 minutes on ice. The solution was incubated in a water bath for 90 seconds at 42°C and was immediately transferred on ice for two minutes. After addition of 500 µl SOC-Medium (see 3.3.1. E) the cells were kept for an hour at 37°C in the shaking incubator (Innova 4300, New Brunswick Scientific). Subsequently, the solution was plated onto a LB-Agar plate (see 3.3.1. G). Following several hours under laminar air flow at room temperature the solution was incubated overnight at 37°C (CO_2-incubator, BB6220, Heraeus Instruments). The next day, about twenty colonies were picked. Each of the colonies was supplied with 5 ml LB medium (see 3.3.1 C) and incubated in the shaking incubator overnight at 37°C. Several of the colonies were tested to verify the point mutation. For this purpose they were incubated with an appropriate restriction enzyme (see e.g. 4.1.3) and separated by electrophoresis. A putatively correct colony gives bands at the expected lengths (for an example see Fig. 11). This colony was then transferred to 10 ml LB medium (see 3.3.1. C), to which 20 µl ampicillin stock solution were added (see 3.3.1. F). This bacterial suspension was finally incubated in the shaking incubator overnight at 37°C.

3.3.4. Plasmid preparation

On the following day the bacteria were separated by centrifugation (Jouan cool centrifuge GR 2022, Rotor AG 100.18, 6000 g, 4°C, 15 min). The pellet was resuspended in 4 ml resuspension buffer (see 3.3.1. J). After addition of 4 ml lysis buffer (see 3.3.1. K) the suspension was cautiously mixed and incubated 5 min at room temperature. 4 ml of neutralization buffer (see 3.3.1. L) were added, carefully mixed and the suspension was incubated 15 mins on ice. After centrifugation of the bacterial lysate (Jouan cool centrifuge GR 2022, rotor AG 16.20, 30.000 g, 4°C, 30 mins), the supernatant was immediately removed and added to a column (Qiagen-tip 20, Nr. 10023, Qiagene) which had been equilibrated with 4 ml equilibrium buffer (see 3.3.1. M). Subsequently, the column was washed twice with 10 ml of washing buffer (see 3.3.1. N) and the plasmid DNA was eluted with 5 ml elution buffer (see 3.3.1. O) into a sterile tube. After precipitation of the DNA with 3.5 ml isopropanol at room temperature and centrifugation (Jouan cooling centrifuge GR 2022, Rotor AG 16.20, 25.000 g, 4°C, 30 min), the DNA precipitate was washed twice in 70% ethanol, centrifuged again and dissolved in sterile Tris/EDTA buffer (see 3.3.1. P).

3.3.5. Sequencing

Point mutations were verified by sequencing which was done by SEQLAB (Göttingen).

3.4 Transfection

3.4.1. Solutions for transfection

A) Diethylaminoethyl dextran stock solution

500 mg of diethylaminoethyl dextran (DEAE dextran, D 9885, sigma) were dissolved in 10 ml bidest. water and autoclaved.

B) TBS buffer (Tris buffered saline)

Solution A:

80 g of sodium chloride (Nr. 1.06404, Merck), 3.8 g of potassium chloride (Nr. 4936, Merck), 2.0 g anhydrous disodium hydrogen phosphate (Nr. 1.06586, Merck) and 30 g Tris (Nr. 100840, Paesel + Lorei) were dissolved in 900 ml bidist. water, adjusted to pH 7.5 with 1 N NaOH and filled up with bidist. water to 1000 ml.

Solution B:

1.5 g calcium chloride (Nr. 2382, Merck) and 1.0 g of magnesium chloride (Nr. 5833, Merck) were dissolved in 100 ml bidist water.

Both solutions were stored at 4°C.

For production of TBS buffer, 100 ml of solution A were mixed with 890 ml bidist. water. Subsequently, 10 ml of solution B were added dropwise under stirring. The solution was sterile filtered (see 3.1.1. B) and stored at 4°C.

C) DNA/DEAE dextran mixture

36.4 µl TBS (3.4.1. B) were mixed with 83.6 µl DEAE dextran stock solution (see 3.4.1. A) and 20 µg plasmid-DNA were added to 60 µl TBS. Subsequently, both solutions were carefully mixed.

D) HBSS buffer (Hepes buffered saline solution)

8 g sodium chloride (Nr. 1.06404, Merck), 0.37 g potassium chloride (Nr. 4936, Merck), 107.7 mg anhydrous disodium hydrogen phosphate (Nr. 1.06586, Merck) and 5 g HEPES (Nr. 242608,

Boehringer Mannheim) were dissolved in 900 ml bidist. water, adjusted to pH 7.05 with 1 N NaOH and filled up with bidist. water to 1000ml. The solution was sterile-filtered (see 3.1.1. B) and stored at 4°C.

E) 10% FCS in TBS

3.6 ml TBS (see 3.4.1. B) were mixed with 0.4 ml of sterile-filtered FCS under laminar air flow. The solution was freshly prepared each time.

F) 10% DMSO in HBSS

Per plate, 4.5 ml HBSS (see 3.4.1. D) were mixed with 0.5 ml autoclaved DMSO (Nr. 1.02952, Merck) under laminar air flow. The solution was freshly prepared each time.

G) Tris buffer pH 7.4 (1 M)

36.3 g Tris (Nr. 100 840, Paesel + Lorei) were dissolved in 200 ml bidist. water, adjusted to pH 7.4 with 37% HCl, filled up with bidist. water to 300 ml and autoclaved.

H) Sodium chloride solution (5 M)

2.92 g of sodium chloride (Nr. 1.06404, Merck) were dissolved in 10 ml bidist. water and the solution was then autoclaved.

I) Chloroquine stock solution

51.6 mg chloroquine (Nr. C-6628, Sigma) were dissolved in 9.22 ml bidist. water and mixed with 0.5 ml 1 M Tris buffer (see 3.4.1 G) pH 7.4 and 0.28 ml of 5 M sodium chloride solution (see 3.4.1. H). This solution was then sterile-filtered (see 3.1.1. B), split up in to aliquots and stored in the dark at - 20°C.

J) DMEM/100 µM chloroquine/2% FCS medium

Per plate, 200 µl FCS, 100 µl chloroquine stock solution (see 3.4.1. I) and 9.7 ml DMEM (see 3.1.1. B) were mixed. The solution was freshly prepared under laminar air flow each time.

3.4.2. Transfection by means of the DEAE dextran method

The transient transfection of COS7 cells (see 3.1.2.) was carried out by means of a modified DEAE dextran method. 24 hours before transfection COS7 cells were plated at a density of 500000 cells per 94 mm Petri dish (see 3.1.1. B) and allowed to attach overnight. The next day, DNA/DEAE dextran mixture (see 3.4.1. C) was prepared and incubated 30 min at room temperature. During this incubation the FCS solution in TBS (see 3.4.1. E) was prepared and the cells were washed twice with 8 ml HBSS (see 3.4.1. D). The DNA/DEAE dextran mixture was diluted with 4 ml of the freshly prepared FCS solution in TBS, cautiously mixed and added to the washed cells. The plates were incubated under 5% CO_2 for four hours at 37°C (CO_2 incubator BB6220, Heraeus Instrument). During this time DMSO solution in HBSS (see 3.4.1. F) and chloroquine medium (see 3.4.1 J) were prepared. The chloroquine medium was protected against light and both solutions were kept at 37°C.

Subsequently the DNA/DEAE dextran solution was removed and 5 ml of the warmed DMSO solution (see 3.4.1. F) were added. After 2 min of incubation at room temperature the DMSO solution was sucked off and the chloroquine medium was added. After few hours of incubation at 37°C under 5% CO_2 the cells were washed twice with 8 ml HBSS and 12 ml culture medium (see 3.1.1. B) were added.

3.5. Membrane preparation

3.5.1. Solutions

A) Phosphate buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 6.5 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 0.7 mM)

12 g sodium chloride (Nr. 1.06404, Merck), 300 mg potassium chloride (Nr. 4936, Merck), 1.725 g anhydrous disodium hydrogen phosphate (Nr. 1.06586, Merck), 150 mg magnesium chloride (Nr. 5833, Merck) and 150 mg calcium chloride (Nr. 2382, Merck) were dissolved in 1000 ml bidist. water. The pH-value was adjusted with 1 N HCl to 7.4 and the volume was filled up with bidist. water to 1.5 l.

B) Phenylmethylsulfonylfluoride (PMSF 100 mM)

Immediately before use, 8.71 mg PMSF (Nr. P-7626, Sigma) were dissolved in 500 µl 99.9% ethanol.

C) Lysis medium (Tris 5 mM, PMSF 0.1 mM, pH 8.0)

60.5 mg Tris (Nr. 100 840, Paesel + Lorei) were dissolved in 90 ml bidist. water. 100 µl 100 mM PMSF solution (see 3.5.1. B) were added and the pH-value was adjusted with 1 N HCl to 8.0. The volume was filled up with bidist. water to 100 ml.

D) Homogenization medium (Tris 50 mM, PMSF 0.1 mM, pH 7.4)

303 mg Tris (Nr. 100 840, Paesel + Lorei) were dissolved in 40 ml bidist. water, 50 µl 100 mM PMSF solution (see 3.5.1. B) were added and the pH-value was adjusted with 1 N HCl to 7.4. The volume was filled up with bidist. water to 50 ml.

E) Tris buffer (5 mM)

60.5 mg Tris (Nr. 100 840, Paesel + Lorei) were dissolved in 90 ml bidist. water and the pH-value was adjusted with 1 N HCl 7.4. The volume was filled up with bidist. water to 100 ml.

3.5.2. Preparation of membranes from COS7-cells

72 hours post-transfection the culture medium was removed from the culture plates and the cells were washed with 5 ml phosphate buffer (3.5.1. A). After addition of 3 ml phosphate buffer cells were harvested with a cell scraper. The cell suspension was transferred to tappered small tubes and the culture plates were rinsed twice with 2 ml of rinsing buffer. The cells were then sedimented 5 minutes at 100 g (4°C Heraeus Megafuge 1.0 R, rotor 2705, 900 rpm).

The sedimented cells of each tube were resuspended in 500 µl cold lysis medium (4°C, 3.5.1. C), transferred to a 1 ml glass homogenizer (Nr. 19, Kontes Glass Co, Vinelind, NJ, USA) and kept on ice for 30 minutes. Subsequently, the suspension was homogenized mechanically under ice cooling with 40 strokes a Teflon-glass homogenizer (500 rpm). For sedimentation of cell fragments, this homogenate was centrifuged for 30 minutes at 4°C and 38000 g (cool centrifuge GR 2022, rotor 2.20, Jouan). The pellets were resuspended in 100 µl cold homogenization buffer each (3.5.1. D), rehomogenized by a glass homogenizer and resedimented (1 h, 38000 g, as above). This second sediment ("membranes") was resuspended in 60 µl cold 5 mM Tris buffer (3.5.2. E). After rehomogenization with the glass homogenizer aliquots of the suspension were filled into precooled cryo-tubes (Roth) and kept frozen at -196°C in liquid nitrogen until binding studies were performed.

3.6. Protein determination (modified according to Sigma, method Nr. TPRO-562)

3.6.1. Solutions

The following solutions were used for protein determination :

Reagent A:	Bicinchoninic acid solution (Nr. B-9643, Sigma)
Reagent B:	4% (w/v) copper-2-sulfate pentahydrate (Nr. 2790, Merck)
Solution A:	5 ml reagent A and 0.1 ml reagent B well mixed
Protein standard:	1 mg/ml bovine serum albumin (Nr. 11924, Serva)

3.6.2. Procedure

For calibration the protein standard was diluted (see 3.6.1.) so that the solutions contained 0, 200, 400, 600, 800 and 1000 µg BSA/ml.

Determination of protein contents was done in a micro titrating plate (Nr. 82.1581, Sarstedt). For each incubation set, 10 µl diluted protein standard solution or sample solution were added to 200 µl solution A (see 3.6.1.) and incubated for 30 mins at 37°C under shaking (Incubator SLT, model IS 2.10). The samples were then left at room temperature for 5 min and the absorption at λ = 550 nm was determined in a micro titrating plate reader (SLT, model 340 ATTC). Evaluation of the results was done with the "Easy-Fit"-program (version 5.47, SLT).

3.7. Binding experiments with COS membranes

3.7.1 Solutions

Standard buffer (Tris HCl 50 mM)

60.66 g Tris (100 849, Paesel + Lorei) were dissolved in 9000 ml bidist. water. The pH was adjusted with HCl 37% to 7.40. The volume was filled up with bidist. water to 10 l. The buffer was stored at 4°C for up to one week.

3.7.1.1. Stock solution of radio ligand

[^{3}H]Glibenclamide-stock solution (3 nM; 51.4 Ci/mmol)

4 µl [^{3}H]glibenclamide (250 µCi/250 µl, Order Nr. NET 1024, Dupont) were mixed with 40 ml of standard buffer (see 3.7.1.). The concentration of [^{3}H]glibenclamide was controlled by liquid

scintillation counting and corrected if deviation from the set point (33840 dpm/100 µl) was more than 5%.

3.7.1.2. Stock solutions of test substances

For every displacing ligand a stock solution was prepared, split up in aliquotes and stored at -20°C. From these stock solutions, the required diluted solutions were prepared by successive dilutions with standard buffer (see 3.7.1.). In case of diazoxide the dilutions were prepared with bidist. water.

A) Diazoxide stock solution (10 mM)

3.356 mg diazoxide (Essex Pharma, MG 230.7) were dissolved in 1.5 ml 40 mM NaOH.

B) Glibenclamide stock solution (1 mM)

4.94 mg glibenclamide (Hoechst, MG 493) were dissolved under stirring in 10 ml 50 mM NaOH overnight at 37°C.

C) MgATP solution (1mM)

2.25 mg ATP (Boehringer Mannheim, MG 605.2) were dissolved in 2700 µl 1 mM $MgCl_2$.

3.7.2. Preparation of membranes

An aliquot of the membranes kept at -196°C (see 3.5.2.) was thawed at room temperature, resuspended in standard buffer (see 3.7.1.), transferred into a 0.5 ml tube and sonicated on ice 8-10 times for 1 second each (Sonifier B-12, Branson Sonic Power Company; output control: stage 7, duty cycle 25%, non pulsated; equipment: standard resonator). The membrane suspension was diluted with standard buffer (see 3.7.1.) to the required volume.

3.7.3. Binding experiments

3.7.3.1. Principle of the filtration assay

Vacuum filtration is a common procedure for separation of free and bound ligands in radio ligand binding studies (BENNETT, 1978; REPKE and LIEBMANN, 1987). During filtration, however, the radioligand-receptor-complex is subjected radioligand-free washing buffer for a short period.

Therefore, this procedure is recommended only for radioligand-receptor-complexes with dissociation constants in the low nanomolar range (REPKE and LIEBMANN, 1987).

3.7.3.2 Incubation

Binding of [^{3}H]glibenclamide to SUR1

The incubation medium contained [^{3}H]glibenclamide in a concentration of 0.3 nM. Microsomal protein was added in a final concentration of about 5 µg/ml. Non-specific binding was determined by parallel incubation sets which contained 100 nM non-labeled glibenclamide. Incubations were carried out in 2 ml-tubes without lids (Sarstedt) at room temperature. The final concentrations of added glibenclamide or diazoxide are given in the results. They were adjusted by addition of aliquots of the stock solutions (see 3.7.1.2.) to the incubation medium. Incubations were started by addition of microsomal protein and terminated after 60 min by filtration.

3.7.3.3. Filtration and scintillation counting

The filtration equipment consisted of a 2 l-filter flask (connected to a vacuum pump, Vacuubrand) with a set for membrane filters attached on top. Fiber glass filters (Whatman GF/B, 25 mm of diameter) which had been incubated in standard buffer at 4°C for 15 min were used (see 3.7.1.). The incubation mixture of a sample (1 ml) was pipetted onto the filter and the filter was subsequently washed three times with 4 ml of standard buffer cooled to 4°C each (see 3.7.1.). After rinsing, the filter was transferred to a scintillation vial (Mini Vial C, Roth). The [^{3}H]-content of the filter was determined after addition of 4 ml scintillation liquid (Rotiszint 22, Roth) and equilibration (24 h, room temperature) in a liquid scintillation counter (Tri - Carb 2100 TR, Canberra-Packard).

3.8. Modeling

The model structure for the nucleotide binding folds of SUR1 was developed by H.-J. HECHT (National Institute für Biotechnological Research (GBF), Department of Structure Research Braunschweig, Germany) using the crystal structure of the ATP-binding subunit of histidine permease from Salmonella typhimurium as structural template. The program BRAGI (SCHOMBURG and REICHELT, 1988) was used for sequence exchange and the program O (JONES, 1985) for visualisation. The pictures were drawn using MOLSCRIPT (KRAULIS, 1991) and rendered using gl_render (ESSER L, unpublished) and POV-RaY™.

3.9. Data evaluation

3.9.1. Statistics

Results are shown as mean ± SEM for N independent experiments with independent membrane preparations. P values were calculated by the Mann-Whitney U-test with Bonferroni correction for multiple comparisons (WALLENSTEIN et al., 1980), $p < 0.05$ was considered as significant

3.9.2. Specific binding

Specific binding was determined by subtraction of non-specific binding (s. 3.7.3.2.) from total binding.

3.9.3. Equilibrium binding

The following equation was used for analysis of the relationship between substance concentration and binding:

(1) $B = (100-k) / (1+(IC_{50} / [A])^{n}) + k$

B is the specific binding, [A] is concentration of free substance, IC_{50} is the half maximal inhibitory concentration, n is the slope parameter (Hill coefficient) and k is an additive constant.

3.9.4. Dissociation constants

The dissociation constants (K_D) for binding to SUR1 were calculated by use of the equation (CHENG and PRUSOFF, 1973):

(2) $K_D = IC_{50} / 1 + ([L] / K_{D(R)})$

from the IC_{50}. K_D is the dissociation constant of the displacing substance, [L] is the concentration and $K_{D(R)}$ is the dissociation constant of the radioligand.

3.9.5. Determination of diazoxide binding to SUR1

Diazoxide binding to SUR1 was determined by an indirect assay based on the displacement of [^{3}H]glibenclamide as follows:

(3) Diazoxide (%) = (A-B)/(A-C) • 100,

A = [^{3}H] (Dz)/[^{3}H] (control), B = [^{3}H] (Dz + [ATP])/[^{3}H] ([ATP]) and C = [^{3}H] (Dz + $[ATP]_{MAX}$)/[^{3}H] ($[ATP]_{MAX}$). [^{3}H] is the amount of [^{3}H]glibenclamide specifically bound in the absence of further additions (control) or presence of 280 µM diazoxide (Dz) and/or [ATP]; $[ATP]_{MAX}$ = 100 µM.

3.9.6. Determination of ATP-induced inhibition of glibenclamide binding

100 µM MgATP inhibits high affinity binding of 0.3 nM [^{3}H]glibenclamide to wild type SUR1 and it has been shown that this inhibitory effect is lost if ATP binding or hydrolysis in the NBFs is eliminated by point mutations in the Walker motifs (SIEVERDING, 1998a). Thus, in this study, the effect of point mutations was analysed on the inhibitory effect of ATP on [^{3}H]glibenclamide binding. Values are presented as percentage of the inhibitory effect of 100 µM MgATP in wild type SUR1(24 ± 6 %).

3.9.7. Determination of B_{max}

The number of sulfonylurea receptors (B_{max}) was calculated from displacement of [^{3}H]glibenclamide through non-labeled glibenclamide by use of the equation (DEBLASI et al., 1989):

(8) $B_{max} = B_0 \cdot (K'_D + [L]) / [L]$

B_0 is the number of receptors occupied in the presence of [L], K'_D is the apparent dissociation constant for glibenclamide and [L] is the concentration of [^{3}H]glibenclamide (0.3 nM).

4. Results

4.1 Application of the "QuikChange site-directed mutagenesis" method

For characterization of functional domains in SURs the "QuikChange site-directed mutagenesis" method had to be established. With this method about 1-4 bases of the protein can be exchanged, inserted or deleted. Thus it is possible to replace amino acids with others, add new ones or delete any. Moreover, a basis triplet that encodes for an amino acid can be transformed to a stop-codon, so that protein biosynthesis ends at this site and arbitrarily shortened non-functional proteins develop. Through silent-mutations sites for restriction endonucleases can be inserted in order to combine cDNAs of different SURs and develop chimeras among different isoforms.

4.1.1. Principle of the method

The basic procedure (Fig. 8) utilizes a dsDNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation and a restriction site. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of *Pfu* DNA polymerase. On incorporation of the oligonuclotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with the DpnI restriction enzyme. The *Dpn* I endonuclease (target sequence: 5'–G^{m6}ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and select for mutation-containing synthesized DNA. DNA isolated from almost all Escherichia coli (E. coli) strains is dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA incorporating the desired mutations is then transformed into E. coli (i.e., XL1-Blue supercompetent cells). The transformation plates are incubated for > 16 hours at 37°C. Between 50 and 800 colonies should be obtained.

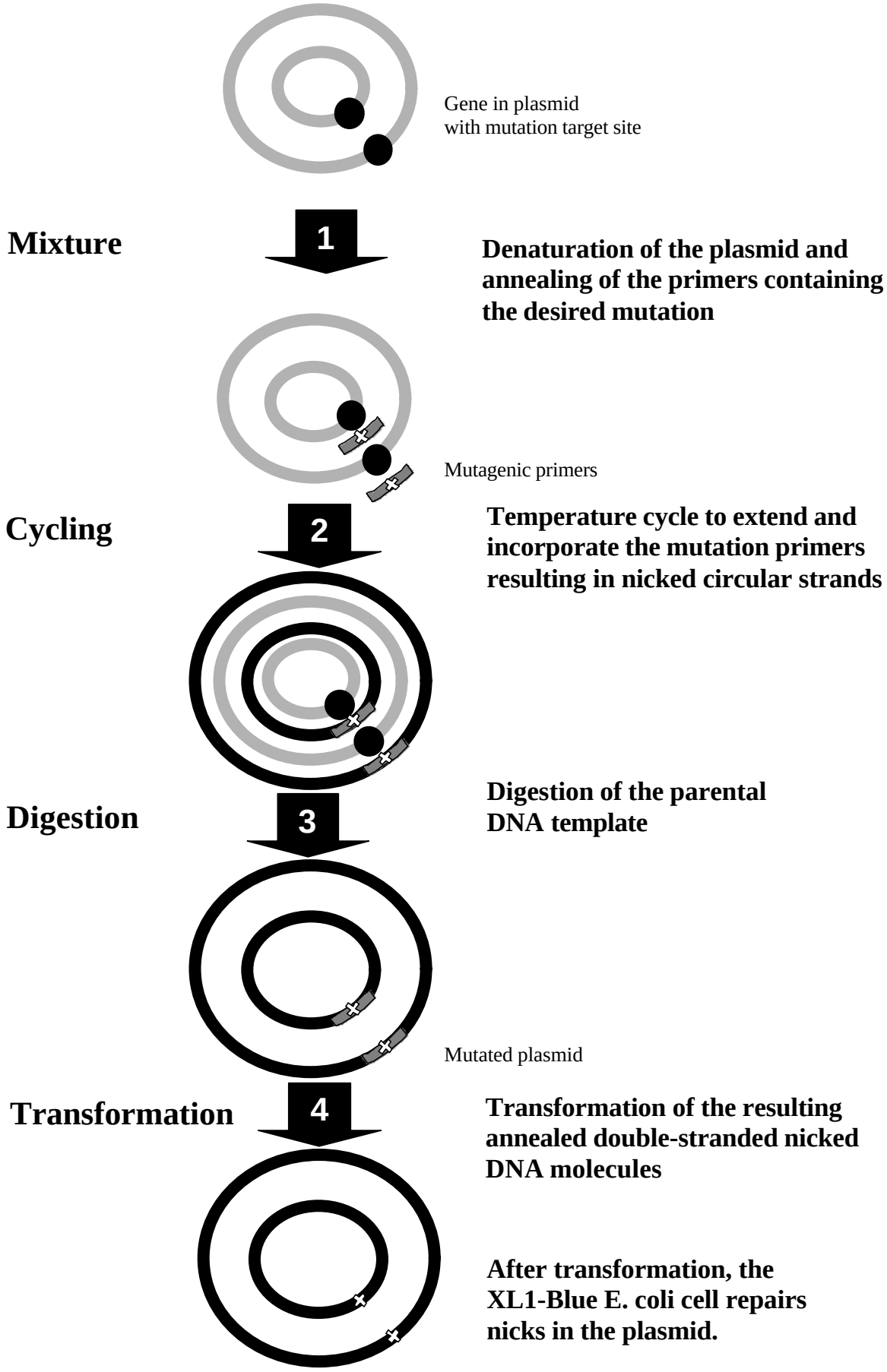


Fig. 8 Overview of the QuikChange™ site directed mutagenesis method. For details see text.

4.1.2. Design of mutagenic oligonucleotide primers:

Mutagenic primers introduce specific experimental mutations. They must be designed individually according to the desired mutation. The following considerations should be made :

1. Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. They should contain the restriction site of certain restriction endonucleases.
2. Primers should be between 25 and 45 bases in length, and their melting temperature (*Tm*) should be ~10°C above the extension temperature of 68°C. The following formula is commonly used for estimating the *Tm*:

   $$Tm = 81.5 + 0.41(\%GC) - 675 / N - \%\ \text{mismatch}$$

   N is the primer length in base pairs, %GC is the ratio of guanine and cytosine to thymine and adenine in the primer and % mismatch is the number of changed bases divided by the the number of bases in the primer.
3. The desired mutation (deletion or insertion) should be in the middle of the primer with ~10-15 bases of correct sequence on both sides.
4. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

In the present study point mutations were introduced into different parts of SUR1. The generation of one of these point mutations is described here as an example, namely the replacement of alanine in position 843 of SUR1 by aspartic acid (A843D). For this purpose the cytosines (C) at positions 2552 and 2553 of hamster SUR1 cDNA were exchanged in order to convert the triplet GCC encoding alanine to GAT encoding aspartic acid (Fig.9). This change simultaneously generated a second Bgl II restriction site (AGATCT).

Subsequently, PCR was carried out as described (3.2.2) and the PCR product was transformed using XL1-Blue supercompetent bacteria. To find a colony with the desired mutation, the DNA of at least 20-30 single bacterial colonies was treated individually by the restriction enzyme Bgl II. The treated DNA was then separated on an agarose gel by electrophoresis and the resulting bands were stained with ethidium bromide. While cutting of the wild type plasmid by Bgl II yielded two fragments of 6554 and 1250 bp in length, cutting of the mutant produced three (5253, 1301 and 1250 bp; Fig.10).

A Wild type sequence (2533 - 2568)

5' **CGTATCAGTGTGGCCAGAGCCCTCTACCAGCAGACC** 3'

3' **GCATAGTCACACCGGTCTCGGGAGATGGTCGTCTGG** 5'

B Mutation sequence (2533 - 2568)

5' **CGTATCAGTGTGGCCAGAGATCTCTACCAGCAGACC** 3'

3' **GCATAGTCACACCGGTCTCTAGAGATGGTCGTCTGG** 5'

C Mutagenesis primer1:

5' **CGTATCAGTGTGGCCAGAGATCTCTACCAGCAGACC** 3'

D Mutagenesis primer 2:

5' **GGTCTGCTGGTAGAGATCTCTGGCCACACTGATACG** 3'

Fig. 9 PCR primers for the mutation A843D in hamster SUR1. **A** = adenine, **C** = cytosine, **G** = guanine, **T** = thymine; mutated bases are underlined.

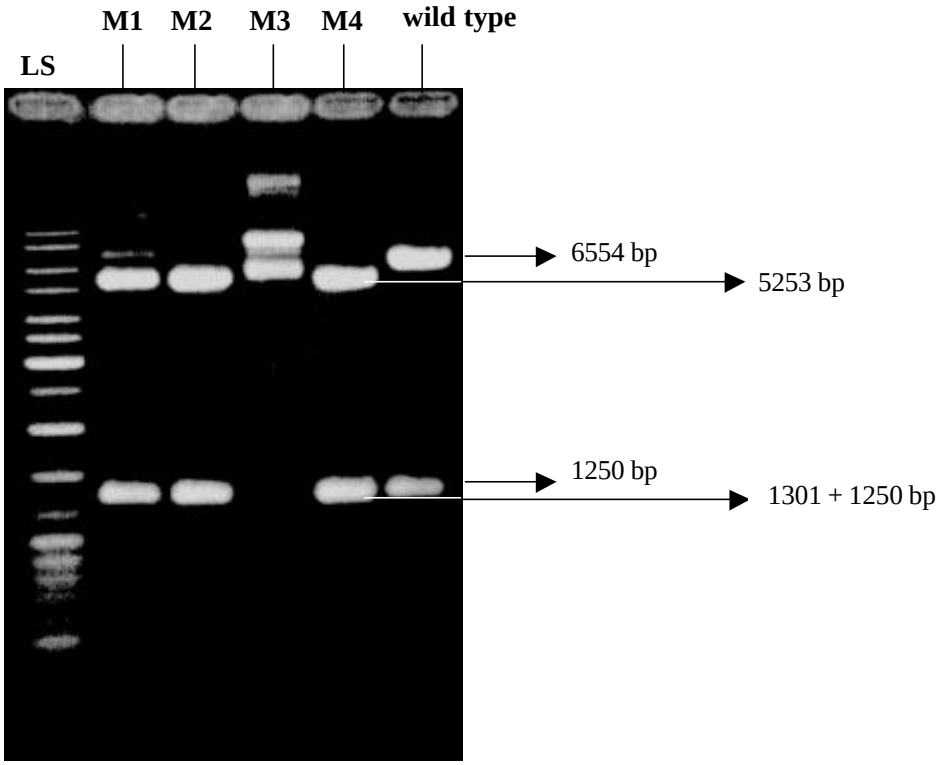


Fig. 10 Separation of the PCR products of mutant (M1-M4) and wild type SUR1 treated with Bgl II *(see 3.3.3).* The treated DNA was separated on an agarose gel against a length standard (LS). The gel was stained by ethidium bromide and evaluated on an UV-transilluminator at 312 nm. Mutant M1 was separated into four bands, therefore it was neglected. Mutants M2 and M4 were separated into three fragments of 5253, 1301 and 1250 bp in length (the bands at 1301 and 1250 bp overlap), whereas the wild type was separated into two fragments of 6554 and 1250 bp in length. One of the correct mutants (M4) was chosen for plasmid preparation (see 3.3.4).

4.2. Functional consequences of point mutations in SUR1 known to induce PHHI

The SUR1 gene consists of 39 exons and 38 introns. Up to now more than 40 mutations in SUR1 are known to induce PHHI (THOMAS et al., 1995 and 1996; NICHOLAS et al., 1996; NESTOROWICZ et al., 1997 and 1998, OTONKOSKI et al., 1999 and SHARMA et al. 2000). Several of these known PHHI-inducing mutations are either insertions or deletions or reside in introns resulting in splice-site or frame shift or nonsense triplets, which lead to truncated proteins with complete loss of K_{ATP} channel activity (Fig. 11).

In this work the functional consequences of 24 point mutations residing in exons (22 PHHI-inducing mutations and 2 polymorphisms) were analyzed. While 12 of these mutations cumulate in NBF2, the rest distributes all over the protein with one mutation in NBF1 (Fig. 11). The mutations were introduced into SUR 1 (see 3.2.2) and the mutant proteins were tested for high affinity [^{3}H]glibenclamide binding, the inhibitory effect of ATP on [^{3}H]glibenclamide binding and for diazoxide binding.

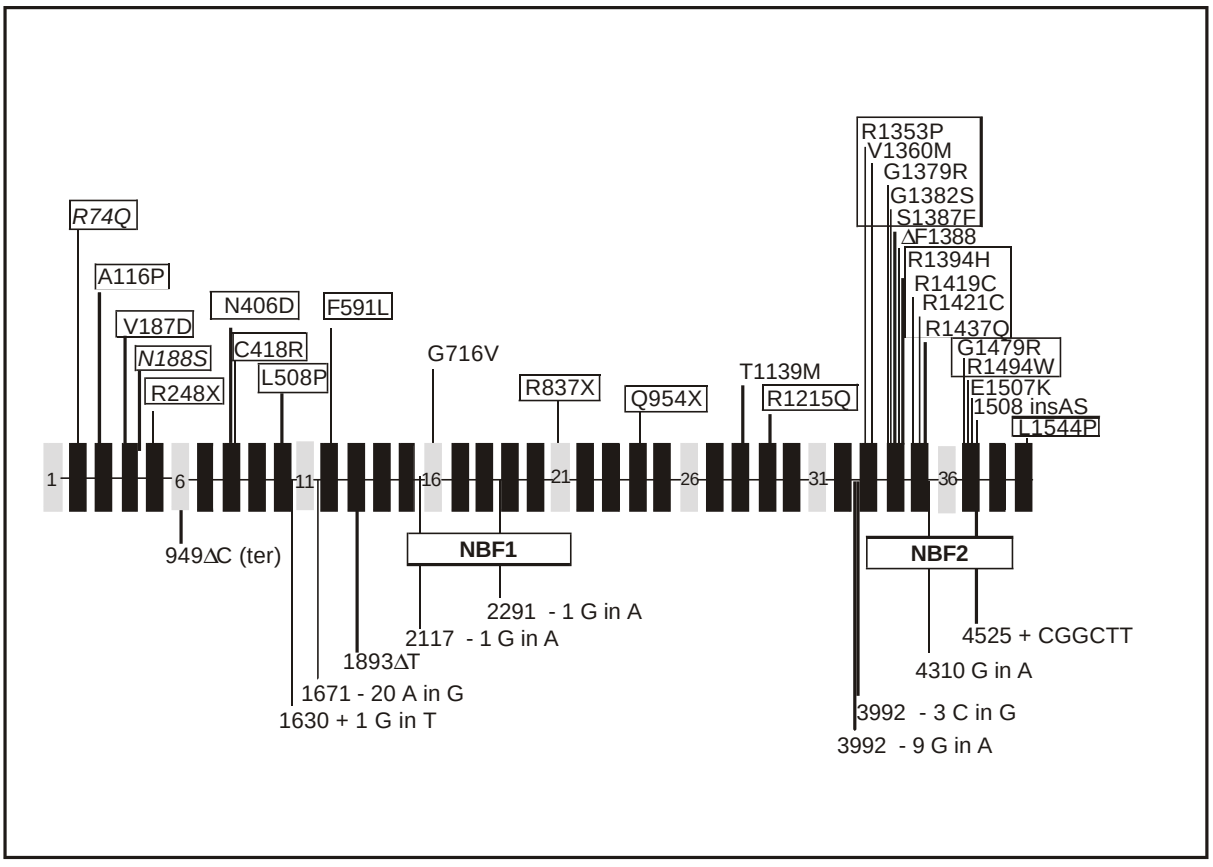


Fig. 11 Scheme of the SUR1 gene. The gene consists of 39 exons represented by filled boxes, and 38 introns represented by the lines connecting the boxes. The scheme illustrates the position of 37 mutations known to induce PHHI and two polymorphisms (R74Q and N188S). 24 mutations residing in exons (in boxes) were introduced into SUR 1 and were functionally analyzed.

4.2.1. Analysis of high affinity [^{3}H]glibenclamide binding

Nine of the constructs (V187D, R248X, R837X, Q954X, R1215Q, G1379R, G1382S, R1394H and R1421C) did not show any high affinity [^{3}H]glibenclamide binding, suggesting that the mutations did either induce truncation or incorrect folding of the protein. For those 15 remaining mutations showing high affinity binding of [^{3}H]glibenclamide K_D values and number of binding sites were determined. None of the mutations exerted a significant effect on the K_D or a clear effect on the expression rates, which means that all B_{max} values lay within the range defined by the 5% and 95% quartil, which was observed for the expression of wild type SUR1 (Fig. 12).

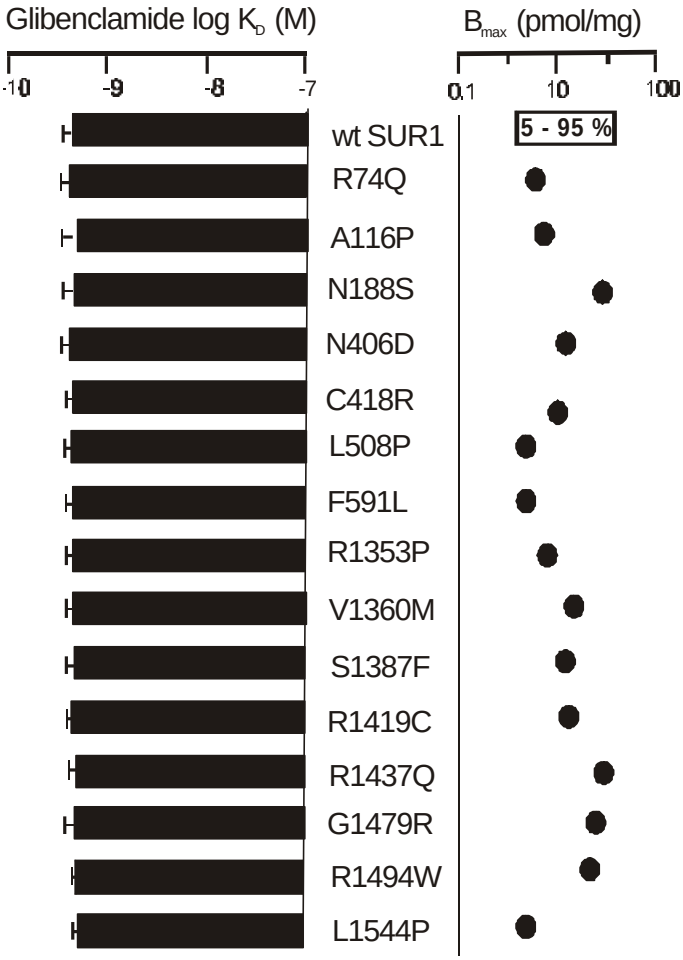


Fig. 12 Dissociation constants and B_{max} values for binding of glibenclamide to wild type and mutant SUR1 isoforms. [^{3}H]glibenclamide (0.3 nM) displacement assays were done with membranes from COS7 cells expressing wild type or mutant hamster SUR1. All incubations were performed in tris-buffer (50 mM; pH 7.4) containing 0.3 nM [^{3}H]glibenclamide and increasing concentrations of non labelled glibenclamide. Incubations were started by addition of COS membranes (about 5µg/ml protein end concentration) and terminated by filtration after 60 minutes. Non specific binding was determined in parallel incubations with 0.1 µM non labelled glibenclamide and specific binding was calculated by subtraction of non specific from total binding, The dissociation constants (K_{Ds}) were calculated according to CHENG and PRUSOFF (1973). The number of sulfonylurea receptors (B_{max}) was calculated from displacement of [^{3}H]glibenclamide by non-labeled glibenclamide (see 3.9.6.) (DEBLASI et al., 1989). Results are shown as mean (± SEM for K_D values) from 4 to 6 independent displacement curves.

4.2.2. PHHI mutations with complete loss of diazoxide binding

Diazoxide is a potent activator of SUR1/K_{IR}6.2 channels and it has been shown previously that diazoxide binding to SUR1 can be measured indirectly by displacement of high affinity [^{3}H]glibenclamide binding (SCHWANSTECHER et al., 1998). Using that assay (see 3.7.3) four of the mutations (R1494W, R1419C, L1544P, R1437Q) with preserved high affinity glibenclamide binding, were found to completely abolish diazoxide binding ($p < 0.01$, $n = 4–6$; Fig. 13). Interestingly, the mutations did also lead either to an abolishment or a strong reduction of the inhibitory effect of 100 µM MgATP on high affinity [^{3}H]glibenclamide binding ($p < 0.01$, $n = 4–6$; Fig. 13). Notably, three (R1494W, R1419C, R1437Q) of these four mutations reside in NBF2 (Fig. 11).

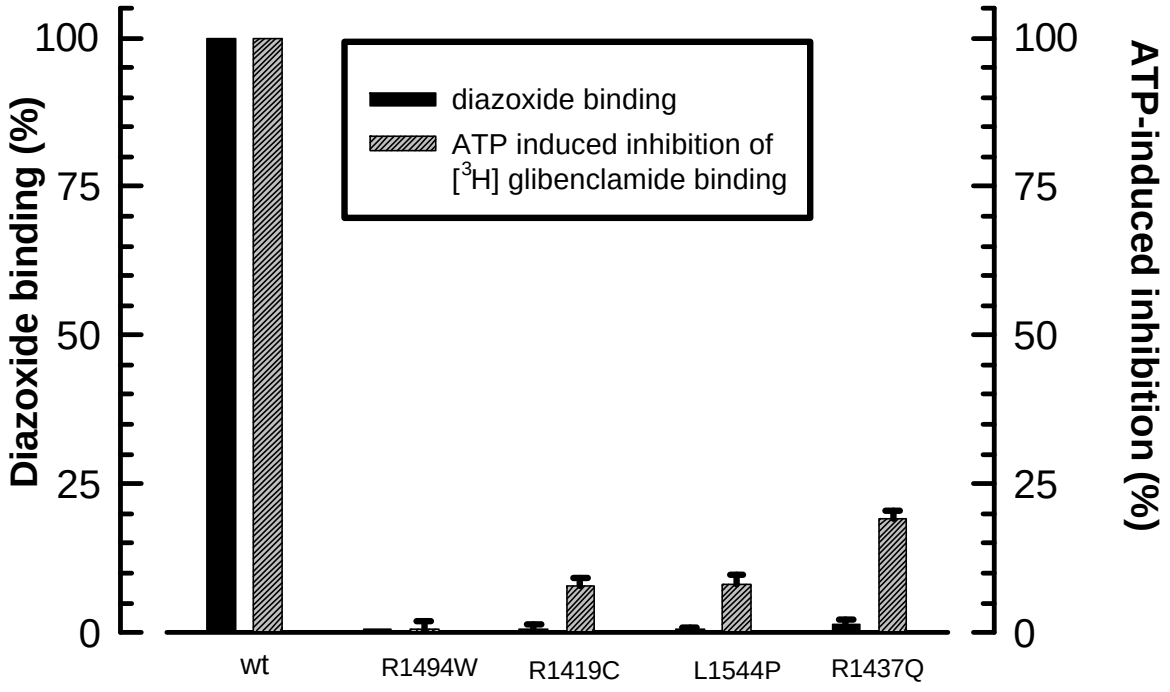


Fig. 13 PHHI- mutations with complete loss of diazoxide binding. Binding assays were carried out with membranes from COS7 cells expressing wild-type or mutant hamster SUR1. The incubation medium contained 0.3 nM [^{3}H]glibenclamide, 280 µM diazoxide, 100 µM MgATP and 1mM free Mg^{2+}. Incubation was started by addition of membranes (final protein concentration 5-50 µg/ml) and terminated by fitration after 60 minutes. Diazoxide binding (%) is the percentage of ATP–dependent displacement of [^{3}H]glibenclamide by 280 µM diazoxide as defined in "Material and methods" (3.9.5). ATP induced inhibition (%) is the percentage of displacement of [^{3}H]glibenclamide by 100 µM MgATP as defined in "Material and methods" (3.9.6). Non-specific binding was determined by parallel incubation sets which contained 100 nM non-labeled glibenclamide and amounted to 5 ± 1% of total binding. Results are presented as mean ± SEM for n = 4-6 independent experiments with independent membrane preparations. For further details see (3.7.).

4.2.3. PHHI-mutations with partial loss of diazoxide binding

Nine of the mutations (F591L, S1387F, R1353P, A116P, L508P, G1479R, C418R, N406D and V1360M), however, did not completely abolish but significantly weakened diazoxide binding and again the effects were paralleled by significant reductions of the inhibitory effect of MgATP on glibenclamide binding ($p < 0.01$ or $p < 0.05$ and $n = 4$-6 each; Fig. 14). Mutations inducing partial loss of diazoxide binding were distributed all over the protein, residing either in transmembrane domains, like A116P, intracellular loops, like C418R or NBF2, which for example is true for S1387F.

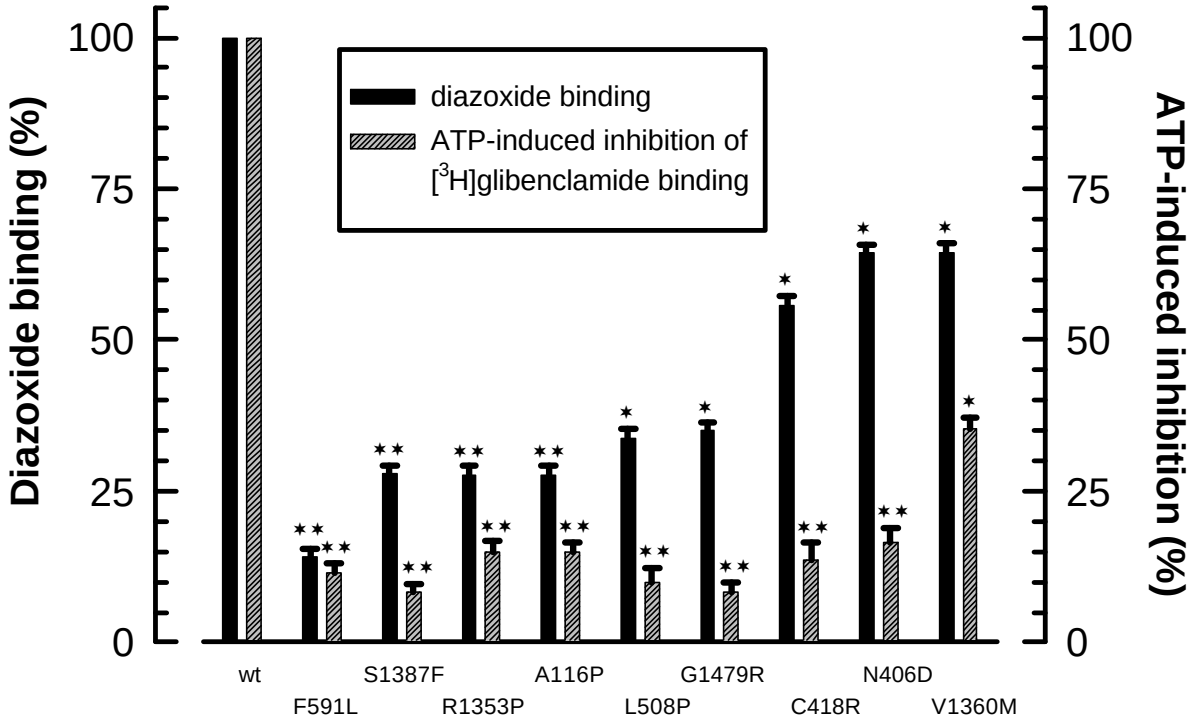


Fig.14 PHHI-mutations with partial loss of diazoxide binding. Results are presented as mean ± SEM for n = 4-6 independent experiments with independent membrane preparations. ⋆⋆$p < 0.01$ or ⋆$p < 0.05$ for comparison with the wild type. For further details see Fig. 13.

4.2.4. Mutations that do not affect diazoxide binding

To analyze whether loss of potassium channel opener binding is a general phenomenon of point mutations in SUR1, R74Q and N188S were tested which presumably represent polymorphisms without functional relevance. In addition to that random mutations were chosen as controls. As shown in Fig. 15, indeed none of these mutations showed a significant reduction of either diazoxide binding or ATP-induced inhibition of glibenclamide binding ($p > 0.35$, $n = 4$-6 each).

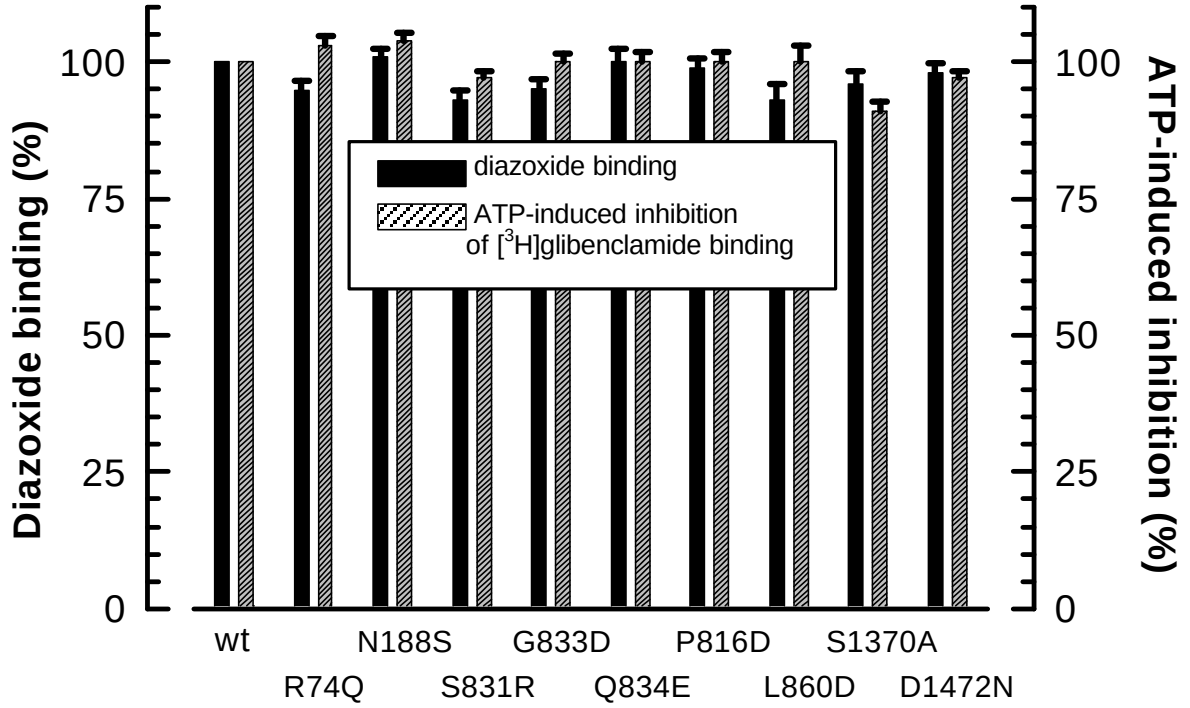


Fig. 15 Mutations that do not affect diazoxide. Results are presented as mean ± SEM for n = 4-6 independent experiments with independent membrane preparations. For further details see Fig. 13.

4.2.5. Correlation of diazoxide binding with the effect of ATP on glibenclamide binding

The effect of all mutations on ATP-induced inhibition of glibenclamide binding and on diazoxide binding was strikingly parallel evidenced by the fact that the square of diazoxide binding showed a strong linear correlation with the effect of ATP on glibenclamide binding (Fig. 16, correlation coefficient = 0.94, $p < 0.0001$).

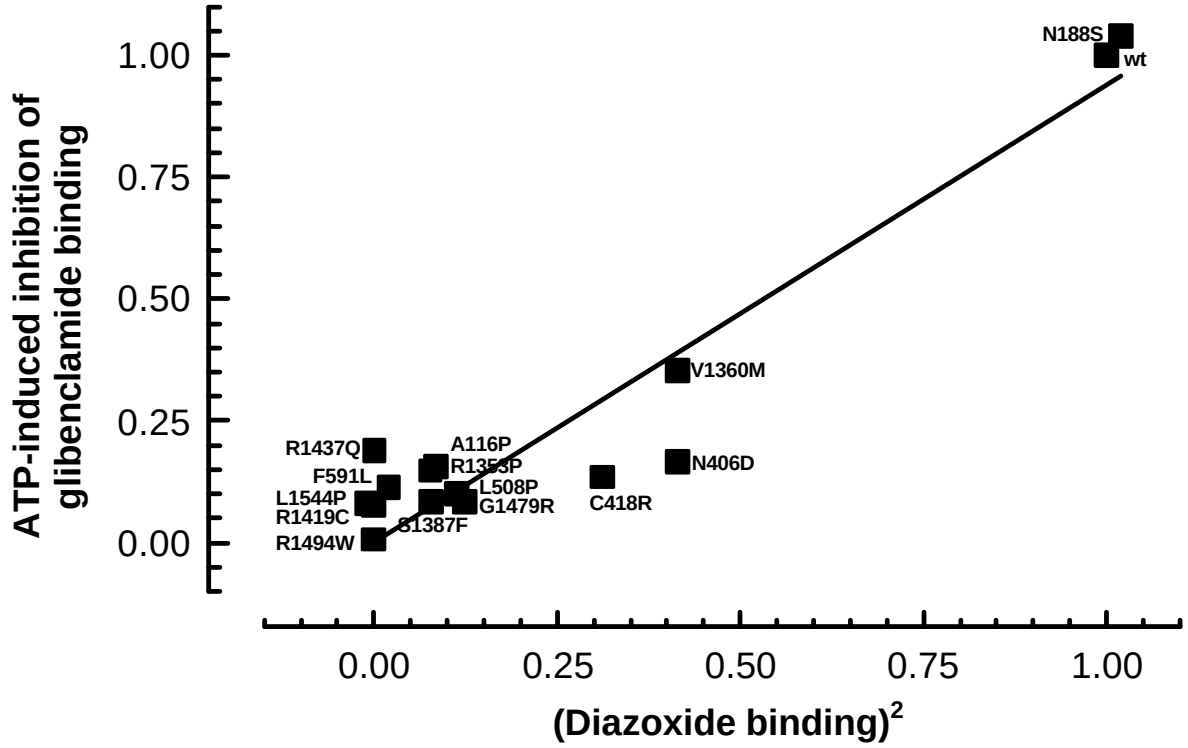


Fig. 16 Loss of diazoxide binding correlates with the inhibitory effect of ATP on glibenclamide binding.

4.3. Homology modeling of the nucleotide binding folds in SUR1

4.3.1 Comparison between the sequences of HisP and the NBFs of SUR1

The recent report of the high resolution crystal structure of HisP, the NBF subunit of the histidine permease complex of Salmonella typhimurium (HUNG et al., 1998), represents a significant breakthrough towards the elucidation of the mechanism of solute translocation by ABC transporters. It was therefore of great interest to find out whether the tertiary structure of the NBFs of SUR1 can be deduced from these data.

Sequence alignment revealed a high degree of homology between HisP and the NBFs of SUR1 with especially strong conservation within the characteristic motifs, i.e. the Walker A and B motifs and the so-called linker region. Particularly, the identical sequence of β-sheets and α-helices (with a total of 12 β-sheets and 9 α-helix-elements in each of the three proteins) pointed at very similar tertiary structures (Fig. 17).

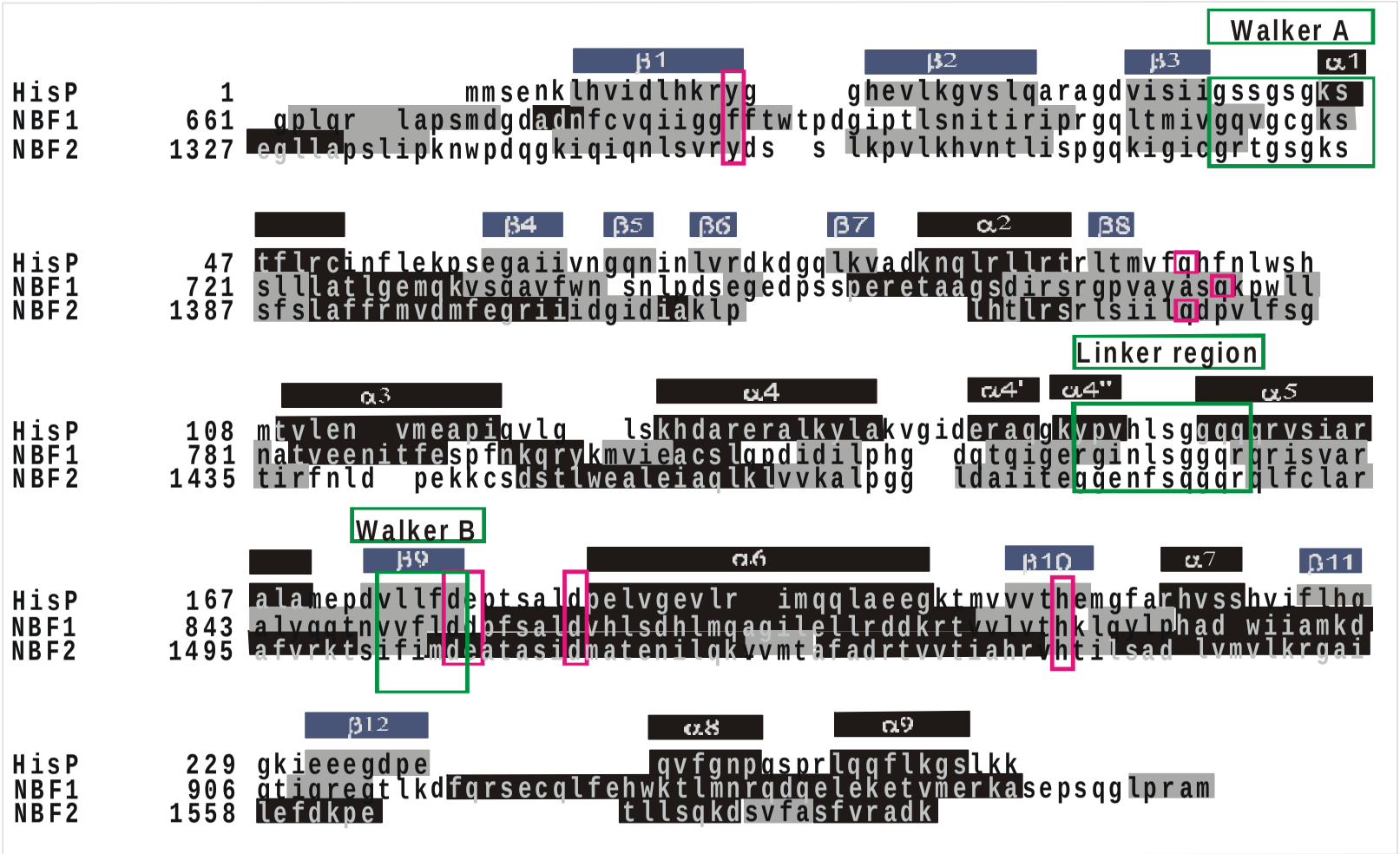


Fig. 17 Sequence alignment of HisP with NBF1 and NBF2 of SUR1. The top rows show secondary structure assignments, the second rows show residue numbers of HisP, the third and fourth rows residue numbers of NBF1 and NBF2 of SUR1, respectively. Heavy shading indicates α-helices whereas light shading indicates β-sheets. The Walker A, Walker B and linker region and well conserved amino acids are boxed. Alignment was carried out with the program "MegAlign" (Laser Gene) using the "Cluster-method".

4.3.2 Analogous model for the tertiary structure of NBF1 and NBF2 of SUR1

Based on the data obtained from the crystal structure of HisP and the sequence alignment described above, a model for the tertiary structure of both NBFs of SURs was deduced. In this model, the structure of either NBF1 or NBF2 is that of an "L" with two thick arms (arm I and arm II); the ATP binding pocket is near the end of arm I (Fig. 18).

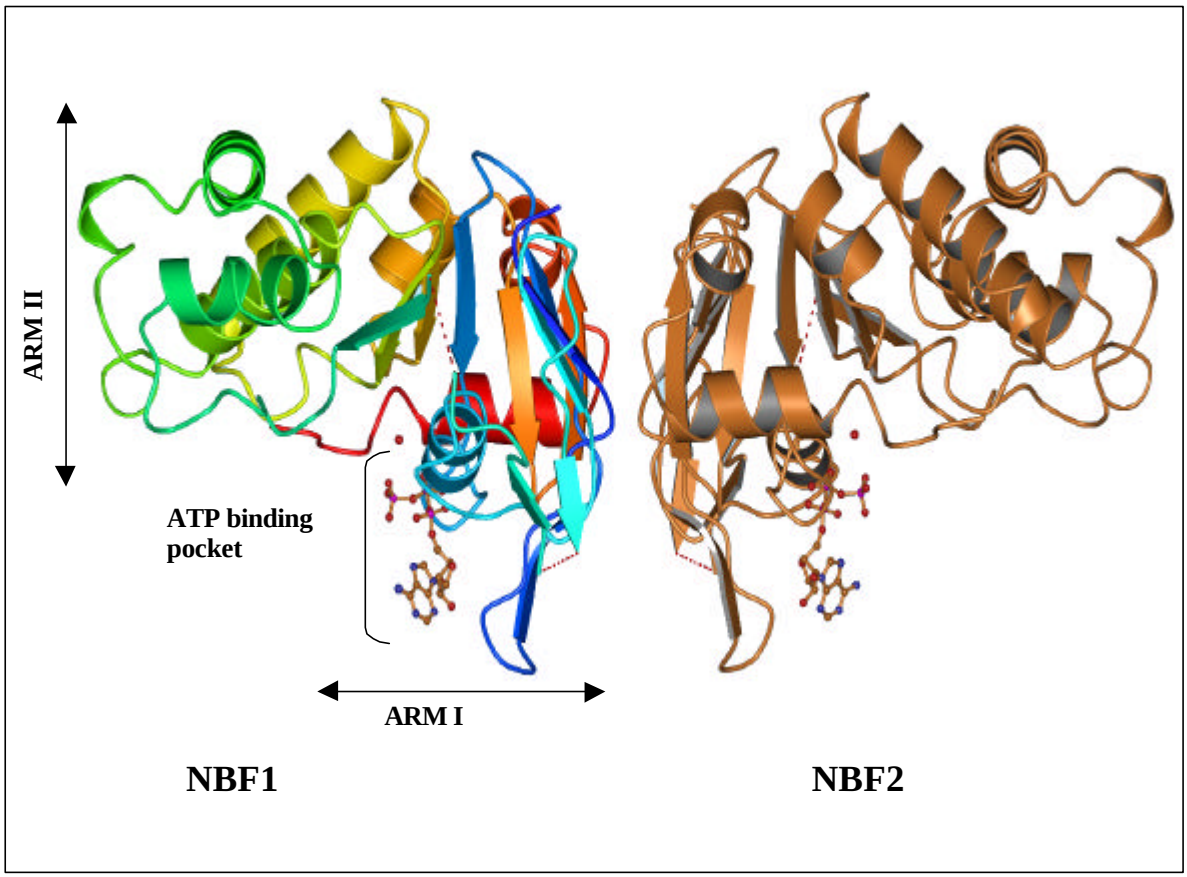


Fig. 18 Analogous molecular modeling of the NBFs of SUR1. View of the NBFs along an axis perpendicular to their two fold axis. The program BRAGI (SCHOMBURG and REICHELT, 1988) was used for sequence exchange and the program O (JONES, 1985) for visualisation. The pictures were drawn using MOLSCRIPT (KRAULIS, 1991) and rendered using gl_render (ESSER L, unpublished) and POV-RaY™. As structural template the coordinates of the ATP-binding-subunit of the histidine permease from Salmonella typhimurium (Brookhaven Protein Databank, code 1bOu) were used.

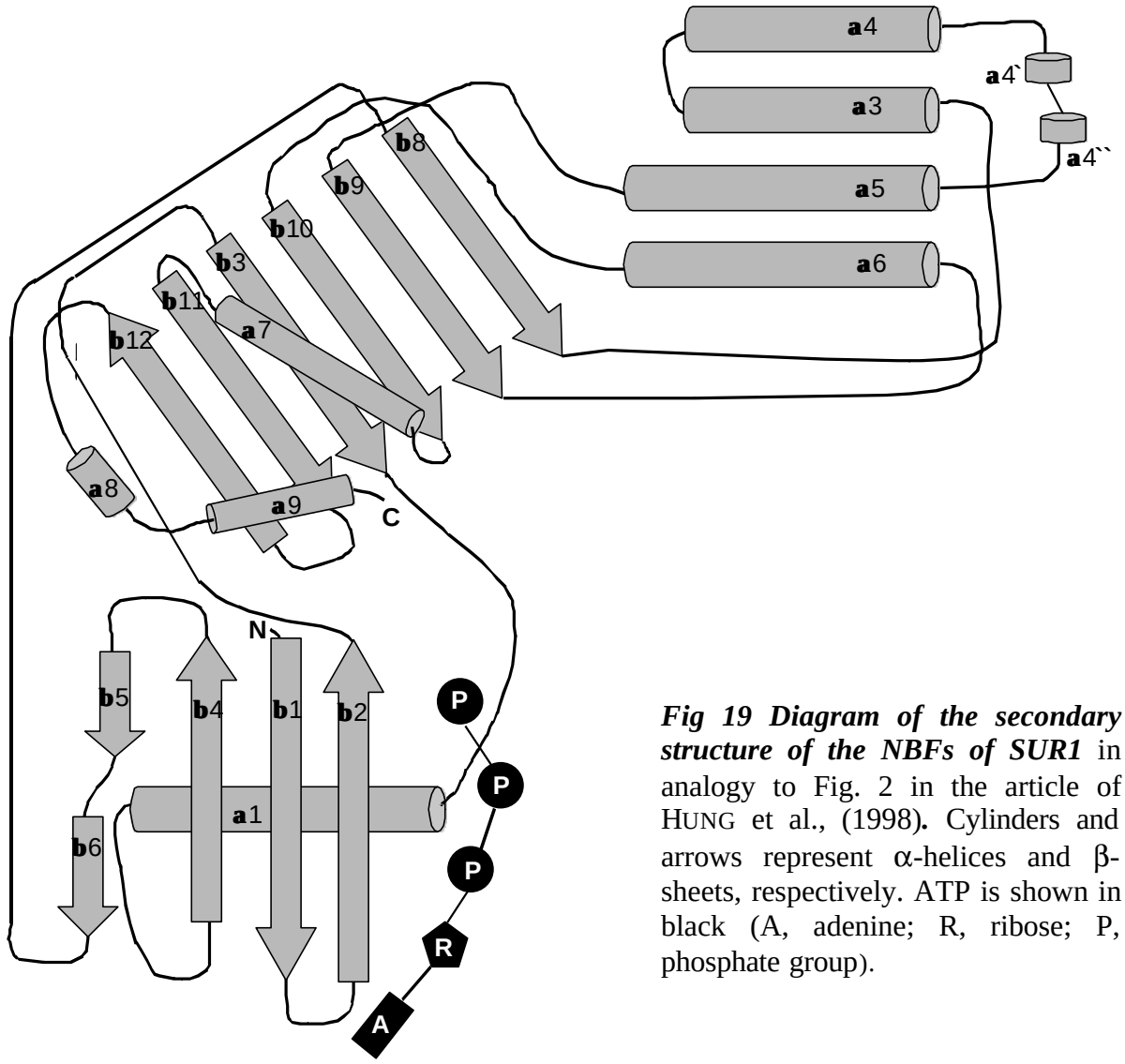


Fig 19 Diagram of the secondary structure of the NBFs of SUR1 in analogy to Fig. 2 in the article of HUNG et al., (1998)**.** Cylinders and arrows represent α-helices and β-sheets, respectively. ATP is shown in black (A, adenine; R, ribose; P, phosphate group).

In analogy to Fig. 2 in the article of HUNG et al. (1998) a diagram of the secondary structure of the NBFs of SUR1 is presented in Fig 19. A six-stranded β-sheet (β3 and β8-β12) spans both arms of the L, with a domain of a α plus β-type structure (β1, β2, β4-β6 and α1) on one side (within arm I) and a domain of mostly α helices (α3-α9) on the other side (within arm II) of the sheet.

4.3.2.1. Interaction of NBF2 with ATP

Figure 20 shows amino acids within NBF2 which were predicted by the model to be critical for ATP-binding. Actually, the tyrosine in position 1354 at the end of β_1 (corresponding to Y16 in HisP) stabilizes the orientation of ATP by way of hydrophobic interactions between the aromatic ring (of tyrosine) and the adenine base (of ATP). The histidine residue (corresponding to H211 in HisP) in the 10th β-sheet supports the orientation of the γ-phosphate via hydrogen bonding. Glutamine 1427 (corresponding to Q100 in HisP) between the 8th β-sheet and the 3rd α-helix as well as glutamic acid 1507 (corresponding to E179 in HisP) in the 9th β-sheet form hydrogen bonds with a water molecule, which interacts with the γ-phosphate of ATP and presumably is of central importance for hydrolysis. Of particular importance also is the aspartate in position 1506 (corresponding to D178 in HisP) at the end of the Walker B motif, which most likely stabilizes the magnesium ion during the hydrolytic cycle (Fig. 20 and 21A).

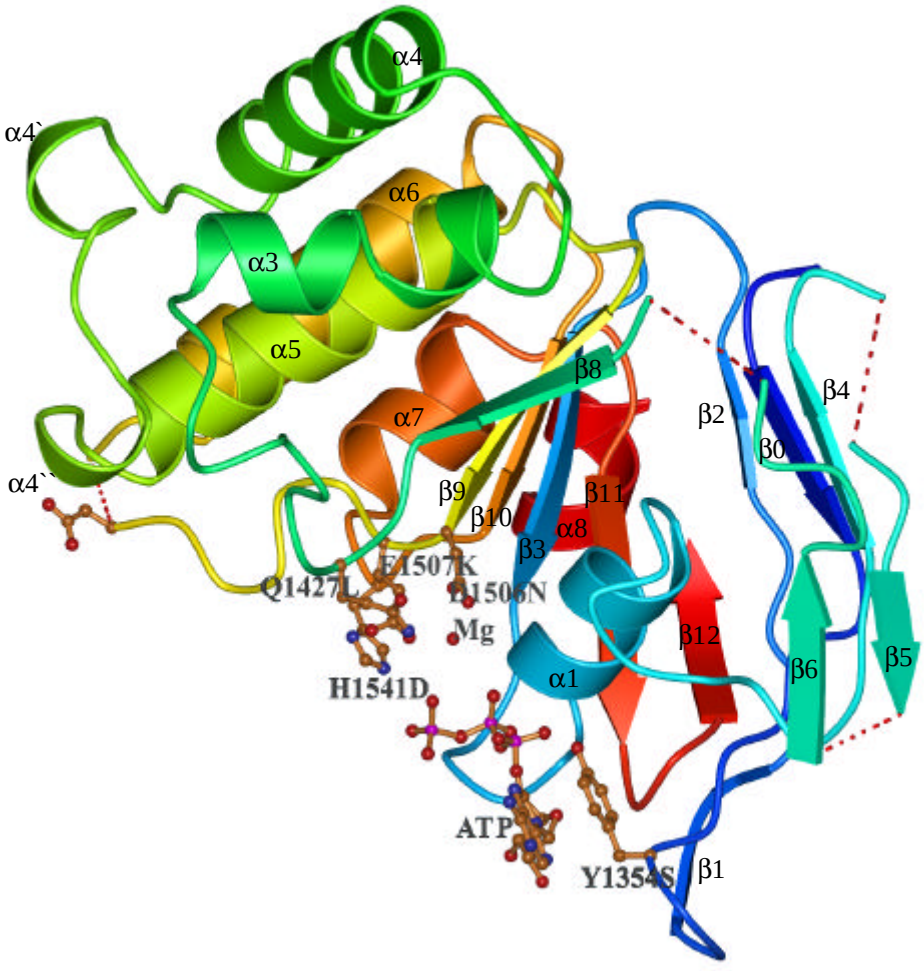


Fig. 20 Conserved amino acids with key importance for ATP-binding to NBF2. For further details see Fig. 18.

To test the functional relevance of these residues point mutations were performed. Corresponding mutations in HisP had been shown to induce partial or complete inhibition of ATPase activity (HUNG et al.,1998).

In accordance with the findings for HisP, substitution of the adenine-ring stabilizing tyrosine in position 1354 by serine led to a complete inhibition of ATP-induced diazoxide binding to SUR1 (Fig. 21). Similarly, complete inhibition of diazoxide binding was observed for substitution of the lysine residue in the Walker A motif (K1385R; see Fig. 21 and Fig. 23). On the basis of the expected model, this residue is of central importance for the coordination of the β-phosphate. Furthermore, substitution of aspartate 1506 (D1506N) as well as substitution of residues involved in coordination of the γ-phosphate through water molecules (Q1427L, E1507K, H1541D) led to partial inhibition of diazoxide binding. This partial inhibition became complete when the substitutions in positions 1427 and 1507 were combined (Fig 21 A and B). Both findings are in good agreement with the predictions made by the model.

4.3.2.2. Interaction of NBF1 with ATP

Similar studies in NBF1 led to somewhat different results. Thus, substitution of the phenylalanine in position 685 (Fig. 22 A) by serine did not have an effect on diazoxide binding, whereas all other substitutions (K719R, D854N, Q775L, D855A and H889D) led to almost complete inhibition of binding.

A

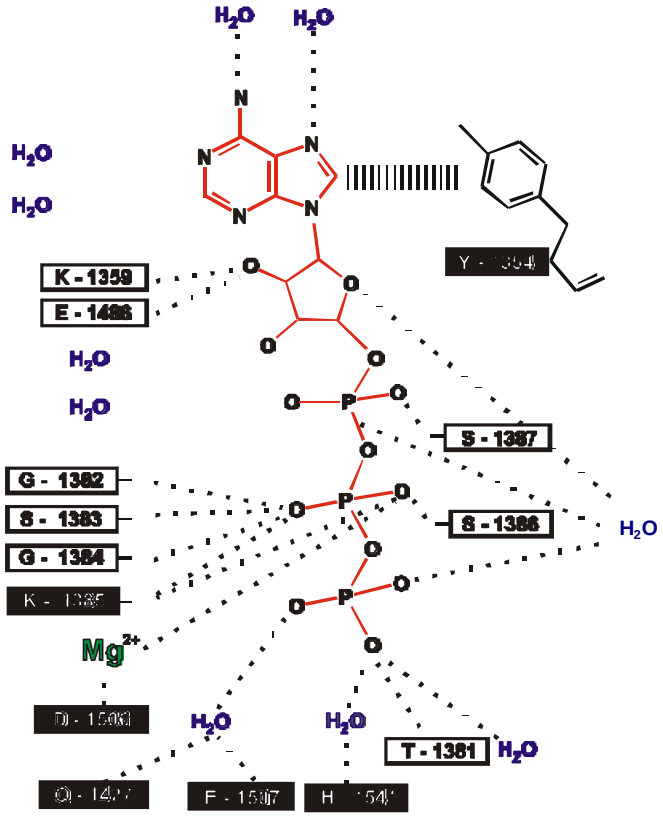
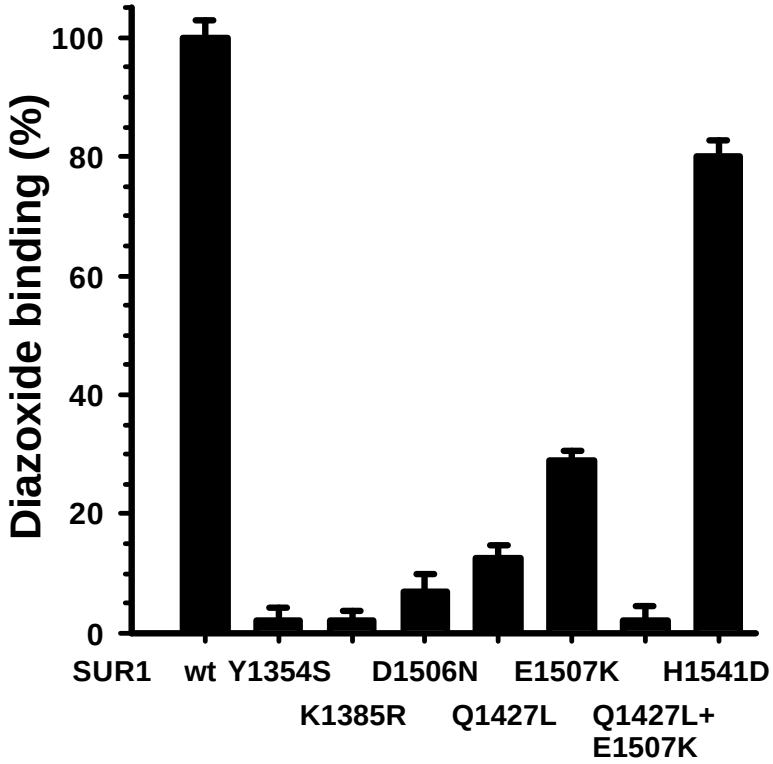


Fig. 21 Interaction of NBF2 of SUR1 with ATP. (A) Atomic details of the putative interaction of NBF2 of SUR1 (residues are boxed) with ATP (centre)**.** Only hydrogen bonds and aromatic stacking are shown, by dashed lines and vertical lines, respectively. The residues investigated (Y1354, K1385, Q1427, D1506, E1507 and H1541) are indicated by black boxes.

B



*(*B) Effect of point mutations in NBF2 on diazoxide binding to SUR1. Binding assays were carried out with membranes from COS7-cells expressing wild type (wt) or mutant SUR1. Mean values ± SEM from n = 5-6 independent experiments are given. For further details see Fig. 13.

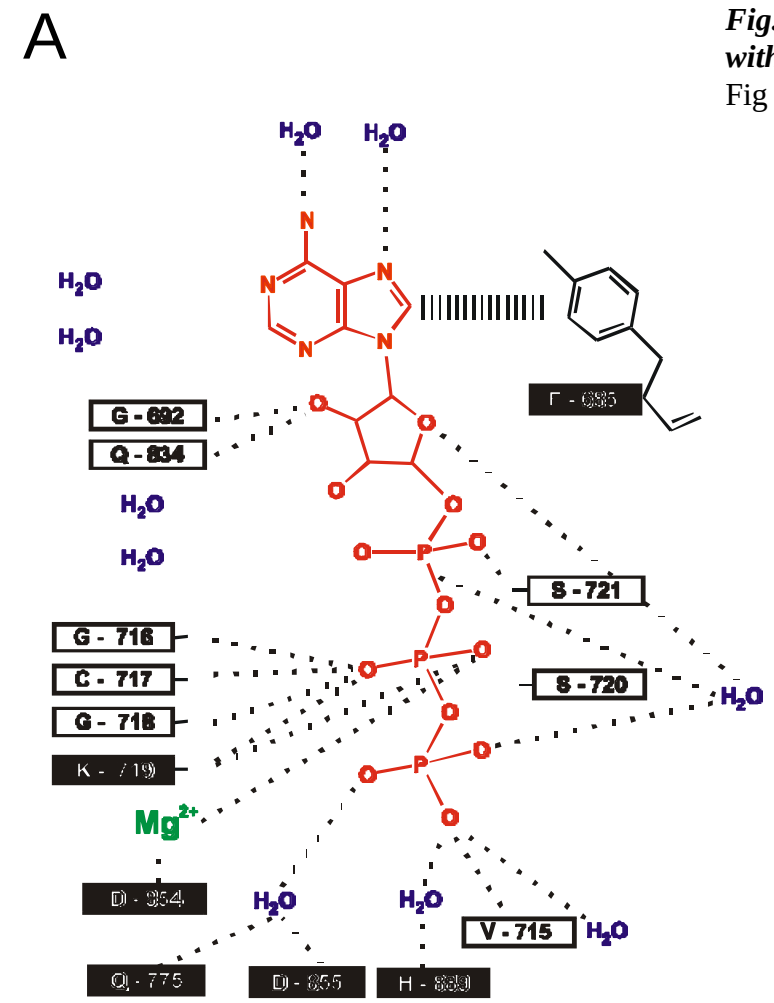
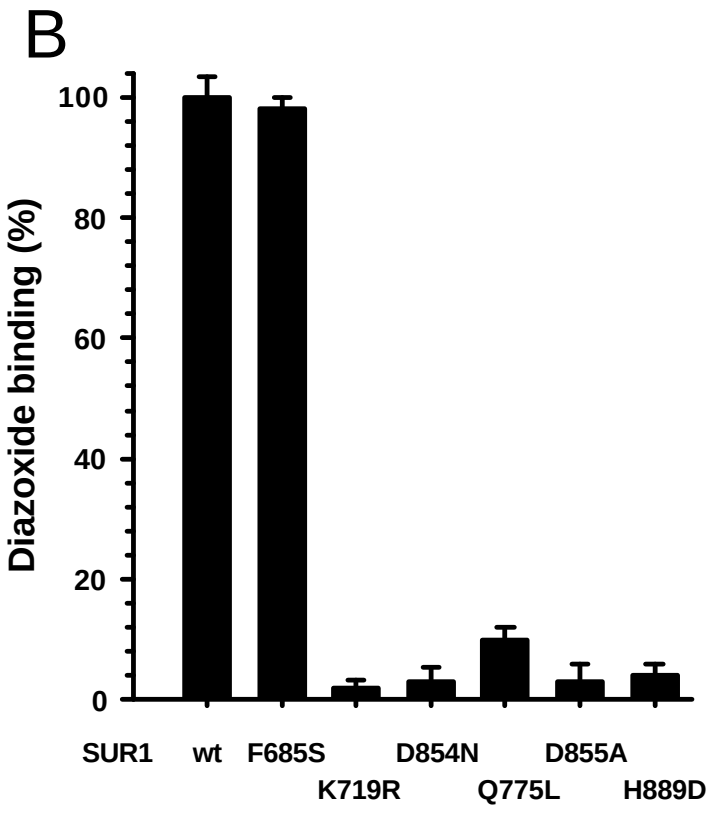


Fig. 22 Interaction of NBF1 of SUR1 with ATP. (A) For further details see Fig 21.



(B) Effect of point mutations in NBF1 on diazoxide binding to SUR1. Binding assays were carried out with membranes from COS7-cells expressing wild type (wt) or mutant SUR1. Mean values ± SEM from n = 5-6 independent ex-periments are given. For further details see Fig. 13.

4.3.2.3. PHHI mutations in NBF2

Various point mutations in NBF2 of SUR1 cause PHHI (see 4.2). Some of them induce a complete loss of high affinity glibenclamide binding, indicating incorrect folding or expression of the protein (see 4.2.1). In others glibenclamide binding is unchanged. However, the latter mutations lead to either an abolishment or a strong reduction of diazoxide binding, indicating that the residues occupy functionally critical positions in NBF2. To verify this hypothesis, the position of PHHI mutations in the presumed tertiary structure of NBF2 of SUR1 was determined. (Fig. 23; for discussion of the results see 5.2)

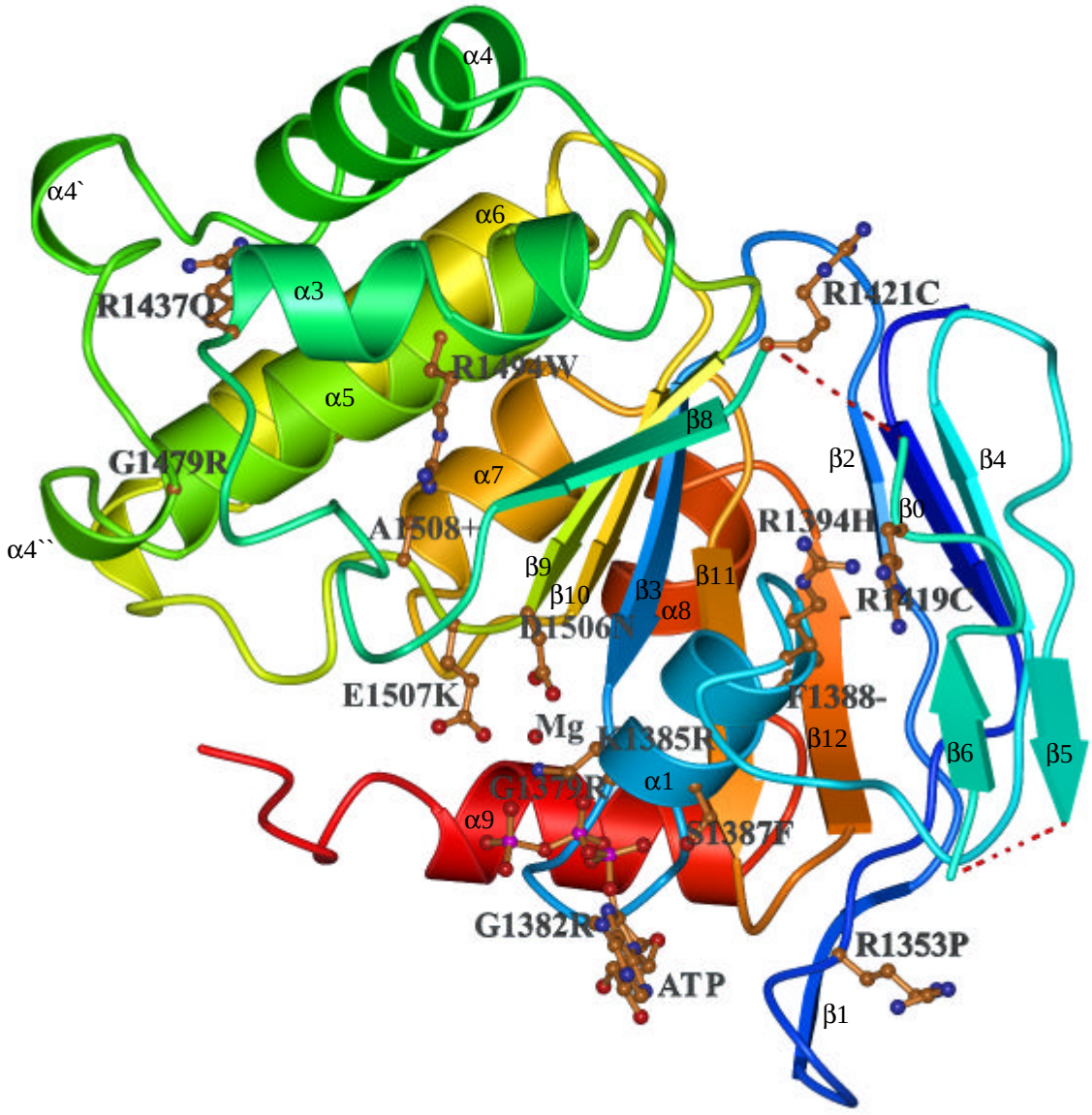


Fig. 23 PHHI mutations in NBF2. K1385R and D1506N are highly conserved residues in the Walker A and B motifs, respectively. For further details see Fig. 18.

5. Discussion

5.1. Loss of KCO-binding is indicative for PHHI

SURs are members of the ATP-binding cassette (ABC) superfamily with two folds (NBFs) for binding and hydrolysis of nucleotides (AGUILAR-BRYAN et al., 1995 and 1998; INAGAKI et al., 1995). These folds have the Walker A and B motifs and a connecting linker region (AGUILAR-BRYAN et al., 1998; HIGGINS, 1992). The Walker A motif, *-gly-x-x-gly-x-gly-lys-(ser/thr)-*, forms part of the nucleotide-binding pocket, with the conserved lysine interacting with the β- and γ-phosphates of ATP (SARASTE et al., 1990; HIGGINS, 1992). Mutation of this residue significantly reduces or abolishes hydrolytic activity in other ATPases (e.g. P-glycoprotein). The linker region with the consensus motif, *-leu-ser-gly-gly-gln-*, is thought to be involved in transducing conformational changes resulting from ATP hydrolysis (AMES et al., 1992).

Mutation of the highly conserved Walker A lysine (K719R or K1385R in SUR1, K711R or K1352R in SUR2B) in either nucleotide binding fold of SUR1 or SUR2B eliminated ATP activation of KCO binding, while binding was weakened, but not abolished by mutations in the linker regions (e.g. G827D or G1479R in SUR1, G809D or G1446R in SUR2B) (SCHWANSTECHER et al., 1998; SCHWANSTECHER, 1998). There was a strong correlation between KCO binding and the activation of mutant SUR1/ or SUR2B/wildtype $K_{IR}6.2$ channels which established that the KCO binding sites on SURs are functionally relevant receptor sites. Mutants without binding activity were not activated by KCOs, while G827D or G1479R in SUR1 and G809D or G1446R in SUR2B showed both impaired KCO binding and KCO activation (SCHWANSTECHER et al., 1998; SCHWANSTECHER, 1998).

The authors therefore concluded that binding and most probably hydrolysis of ATP in both NBFs induce a conformational change, which strongly increases KCO affinity (SCHWANSTECHER et al., 1998; SCHWANSTECHER, 1998). Amazingly, parallel effects on channel activation by the nucleoside diphosphates ADP and GDP were observed (GRIBBLE et al., 1997, SHYNG et al., 1998, SIEVERDING, 1998b; SCHWANSTECHER, 1998), suggesting that the binding sites for KCOs and nucleoside diphosphates might be controlled by the same ATP-dependent mechanism (SIEVERDING, 1998b; SCHWANSTECHER et al., 1998).

Sulfonylurea receptors thus might function as molecular switches (see Fig. 24; SIEVERDING, 1998b; SCHWANSTECHER et al., 1998; SCHWANSTECHER, 1998) - similar to regulatory GTPases (e.g. Ras or EF-Tu; WITTINGHOFER and PAI, 1991, ABEL et al., 1996). In the absence of ATP the receptor is in its insensitive "off conformation" which cannot be

activated by KCOs and NDPs. Hydrolysis of ATP in both NBFs could induce a sensitive conformational state, the "on conformation", which allows interaction of KCOs and NDPs with their receptor sites. Mutations in the NBFs prevent switching, lock the receptor in its "off-conformation" and thus prevent physiological regulation of channel activity by changes in the cytosolic concentrations of nucleoside diphosphates.

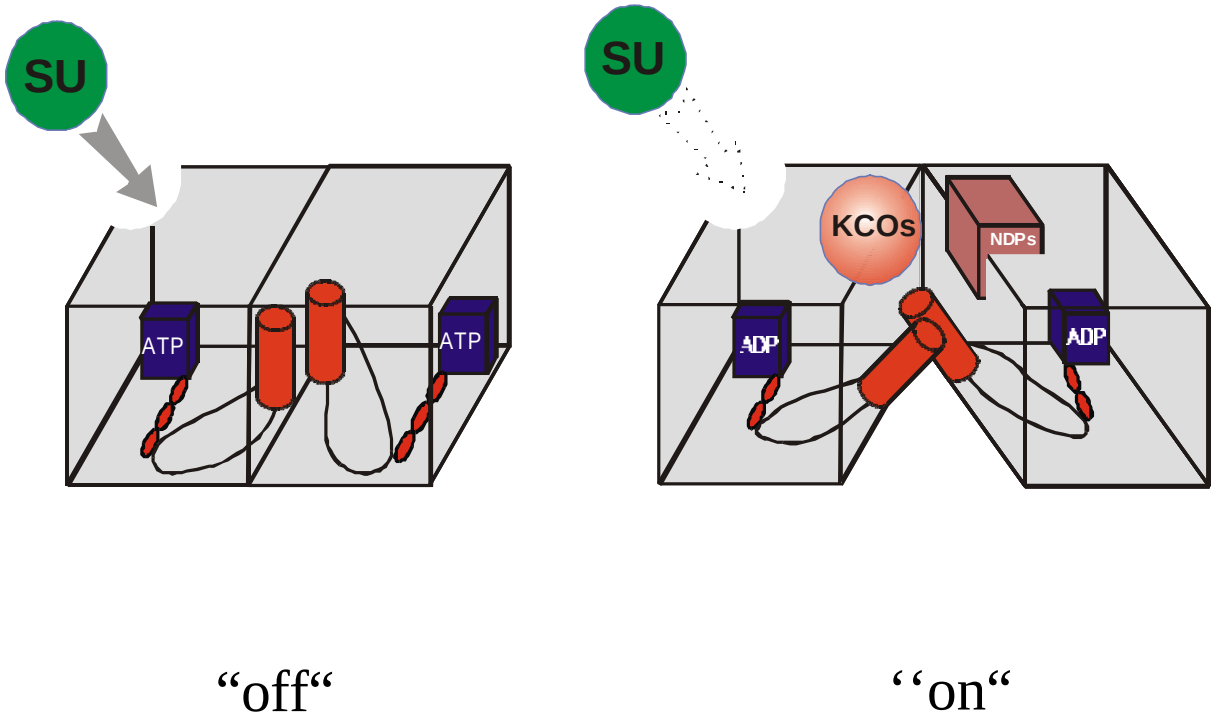


Fig. 24 Sulfonylurea receptors might function as molecular switches. In the absence of ATP the receptors are in their insensitive "off conformation" which cannot be activated by KCOs and NDPs. Binding and presumably hydrolysis of ATP in both NBFs induces a conformational change ("on conformation") which allows interaction of KCOs and NDPs with their receptor sites. The "on conformation" induces a slight decrease in sulfonylurea affinity (Figure according to SIEVERDING, 1998b).

High affinity binding of glibenclamide to SUR1 does not require the "on conformation". Indeed, quite the reverse is true as micromolar concentrations of MgATP induce a reduction of high affinity glibenclamide binding (SCHWANSTECHER et al., 1990, 1991; SIEVERDING, 1998a). A more detailed analysis of the mechanism of ATP-induced inhibition of glibenclamide binding revealed that MgATP did not alter the number of binding sites but slightly reduced glibenclamide affinity by an allosteric interaction (SIEVERDING, 1998a).

In summary, the "on conformation" of the sulfonylurea receptor is characterized not only by a high affinity for KCOs but also by a slightly reduced affinity for sulfonylureas. The "switch model" thus predicts that a loss of KCO-binding paralleled by a loss of an inhibitory effect of MgATP on glibenclamide binding is indicative for a defect in the switch mechanism. Parallel loss of nucleoside diphosphate sensitivity would consequently lead to an inabilitiy of the channel to increase open probability when blood glucose is lowered resulting in hyperinsulinemia and hypoglycemia.

To test this hypothesis 22 mutations in SUR1 known to cause PHHI were functionally analyzed. Nine of the constructs (V187D, R248X, R837X, Q954X, R1215Q, G1379R, G1382S, R1394H and R1421C) did not show any high affinity glibenclamide binding (Fig. 25) suggesting that the mutations did either induce truncation or incorrect folding of the protein resulting in a complete loss of channel activity. Consistently, it was shown previously that V187D, which is the most common PHHI-inducing mutation in the Finnish population, and R1394H did not express measurable K_{ATP}-channel activity (OTONKOSKI et al., 1999; SHYNG et al., 1998).

Functional studies have not been performed for R248X, R837X and Q954X, so far. However, as these three point mutations result in the introduction of a termination codon, the SUR1 proteins encoded by the mutant alleles would be predicted to lack essential parts (see Fig. 24). Thus loss of binding in these mutants is most probably due to the fact that the truncated SURs are unable to form functional channels when coexpressed with $K_{IR}6.2$.

G1379 and G1382 represent the first and second glycine residue of the Walker A motif in NBF2. Both residues are strictly conserved between eucaryotic ABC proteins and substantial evidence exists for the importance of these residues in the structure and function of other members of the ABC superfamily. Consistent with our observation of a complete lack of glibenclamide binding replacement of the corresponding glycine residues in F_1ATPase or histidine permease resulted in either non-functional or dysfunctional molecules (SHEN et al., 1994; SHYAMALA et al., 1991).

While we did not see glibenclamide binding in the R1215Q, G1382S and R1421C (which corresponds to R1420C in human SUR1) mutants, they have been shown by others to generate active channels when coexpressed with $K_{IR}6.2$, albeit the mean current amplitudes were reduced by 40 - 60 % indicating lower expression of the mutant channels (SHYNG et al, 1998, MATSUO et al., 2000). At this stage of our investigations the reason for this discrepancy is unclear.

From the PHHI mutations with preserved high affinity glibenclamide binding four led to a complete abolishment and nine (e.g. F591L, G1479R) to a significant reduction in diazoxide binding (Fig. 14). Consistently, - in other studies - F591L and G1479R were found to induce a substantial impairment of channel activation by diazoxide and nucleoside diphosphates (SHYNG et al., 1998; SCHWANSTECHER et al., 1998; SCHWANSTECHER, 1998).

Neither polymorphisms like R74Q or N188S nor random mutations showed a decrease of diazoxide binding or ATP-induced inhibition of glibenclamide binding (Fig. 15). These findings are consistent with patch clamp studies demonstrating that N188S does not alter the sensitivity of the K_{ATP} channel to nucleoside diphosphates or diazoxide (DUNNE, 1997; SHYNG et al., 1998).

The effects of all mutations on diazoxide binding and ATP-induced inhibition of glibenclamide binding were strictly correlated (Fig. 16). In conclusion, the results from this study strongly support the idea that locking the receptor in its non-binding state induces PHHI via preventing K_{ATP} channel activation by physiological activators, specifically MgADP.

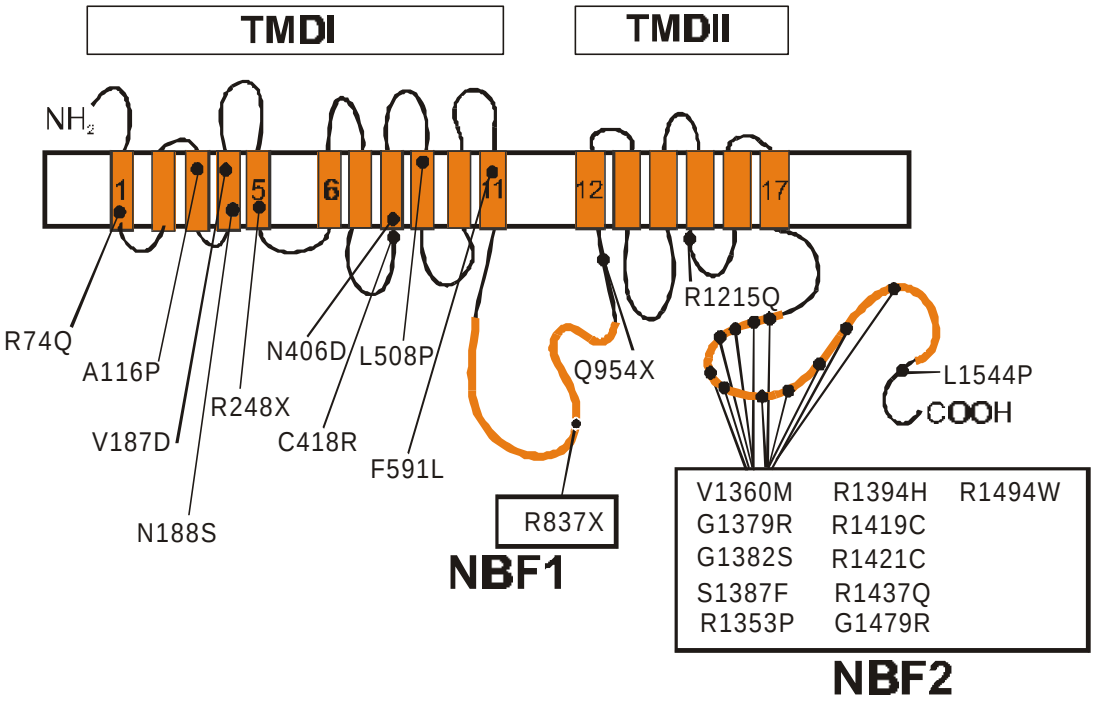


Fig. 25 Putative transmembrane topology of SUR1 as predicted by Tusnady et al., 1997. Positions of PHHI-associated mutations are indicated. V187D, R248X, R837X, Q954X, R1215Q, G1379R, G1382S, R1394H and R1421C did not show any high affinity glibenclamide binding. From the PHHI mutations with preserved high affinity glibenclamide binding four led to a complete abolishment (R1419C, R1437Q, R1494W and L1544P) and nine (A116P, N406D, C418R, L508P, F591L, R1353P, V1360M, S1387F and G1479R) to a significant reduction in diazoxide binding. R74Q and N188S are polymorphisms.

Interestingly, PHHI mutations resulting in loss of potassium channel opener binding were not only localized in NBF1 or NBF2 but also in areas outside the nucleotide binding folds, particularly in transmembrane domains 3, 8, 9 and 11 and the intracellular loops 4 and 8, suggesting that these regions are involved in the switching process.

5.2 The tertiary structure of the NBFs of SURs closely resembles that of HisP

Recently, the three-dimensional crystal structure of HisP, the ATP binding subunit of the histidine permease, has been elucidated (HUNG et al., 1998; see 4.3). Sequence alignment of HisP with the NBFs of SUR1 revealed a high degree of homology and suggested very similar tertiary structures. Based on this alignment and the data from the crystal structure, a tertiary structure of the NBFs of SURs was deduced and the functional relevance of residues predicted to be critical for ATP binding was examined.

Indeed, substitution of highly conserved amino acids in both NBFs involved in stabilizing the orientation of ATP by interacting directly with the β-phosphate (K719 in NBF1, K1385 in NBF2), or indirectly via Mg^{2+} (D854 in NBF1, D1506 in NBF2) or hydrogen bonding with the γ-phosphate (Q775, D855, H889 and V715 in NBF1; Q1427, E1507, H1541 in NBF2) induced a partial or complete loss of diazoxide binding (Fig. 21 and 22). In HisP mutation of the corresponding residues is known to significantly reduce or abolish ATPase activity (SHYAMALA et al., 1991).

Upstream of the Walker A motif of most ABC transporters a conserved aromatic amino acid is found (CROOP, 1998; SCHNEIDER and HUNKE, 1998). The crystal structure of HisP implicates this residue (Y16 in HisP) as a critical binding contact for the adenine ring of ATP and consistently, substitution of tyrosine16 by serine abolished the ability of HisP to bind ATP and consequently disrupted transport (SHYAMALA et al., 1991). In SUR1, Y16 corresponds to tyrosine 1354 in NBF2 and phenylalanine 685 in NBF1. While substitution of Y1354 by serine induced a complete inhibition of diazoxide binding, replacement of phenylalanine 685 was without effect. The failure of F685S to influence diazoxide binding could be due to the fact that this residue is not important for the coordination of the adenine ring in NBF1. However, it is also conceivable that the decrease in ATP-affinity induced by the substitution is not sufficient to reduce diazoxide binding in the presence of 100 µM ATP. This would e.g. be the case if NBF1 had a much higher affinity for ATP than NBF2. Consistent with the latter explanation, a large difference in the affinities of NBF1 and NBF2 has been proposed for CFTR (SENIOR and GADSBY, 1997).

5.3 The tertiary structure of NBF2 in SUR1 predicts the functional consequences of PHHI mutations

Based on our results presented above we would predict various point mutations in NBF2 of SUR1 to cause PHHI by locking the receptor in its "off conformation". Among these R1419C, R1437Q and R1494W led to a complete abolishment and R1353W, S1387F and G1479R to a partial reduction of diazoxide binding (see 5.1). To propose a functional basis for these defects, the mutated residues were localized in the putative tertiary structure of the NBFs of SUR1 (see Fig. 23).

Arginine 1353 presumably stabilizes tyrosine 1354 which is critical for the coordination of the adenine ring of ATP (see 5.2). While glutamate 1507 interacts with the γ-phosphate (see 5.2), serine 1387 most probably stabilizes the α-phosphate of ATP as well as tyrosine 1354 via hydrogen bonding (see Fig. 21 and Fig. 23).

R1419 should be part of a short loop connecting the sixth to the eight β-sheet and thus might be crucial for the conformation of this region which is involved in spanning arm I and arm II (see Fig. 19). R1437 and G1479 are found in a short stretch in front of helical structures (α3 und α5, respectively) forming part of the linker region. Presumably, the orientation of this region is altered by substitution of these amino acids. Moreover, R1494 within the fifth α-helix is part of the linker region which is thought to be involved in transducing conformational changes resulting from ATP hydrolysis (AMES et al., 1992). Indeed, the linker region forms a substantial part of arm II (see Fig. 19 and 23) which in HisP is essential for the interaction between the nucleotide binding folds and transmembrane domains (HUNG et al. 1998).

In conclusion, evidence is presented that the tertiary structure of the NBFs of SURs closely resembles that of HisP and allows to predict the functional consequences of mutations leading to a dysregulation of insulin secretion.

6. Summary

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI), is a disorder of insulin secretion with strong hyperinsulinemia despite severe reduction of blood glucose. At present all defects known to cause this disease finally result in a reduced open probability of the K_{ATP} channel in the plasma membrane of the pancreatic β-cell, thus inducing deregulated membrane depolarisation, opening of L-type Ca^{2+}-channels and insulin secretion triggered by persistently elevated cytosolic Ca^{2+}-levels.

In rare familial cases PHHI is induced by point mutations in the glucokinase or glutamate dehydrogenase gene resulting in either increased affinity for glucose or reduced allosteric inhibition by GTP. Both types of defects lead to an elevation of the cytosolic ATP/ADP ratio with subsequent inhibition of K_{ATP} channel activity. Defects in the K_{ATP} channel itself, however, seem to be a much more common cause for the syndrome and up to now more than 40 mutations in SUR1 are known to induce PHHI. Some of these mutations are either insertions or deletions or reside in introns resulting in splice- site or frame shifts or nonsense triplets, which lead to truncated proteins with complete loss of K_{ATP} channel activity. Others, however, induce a reduction of the response to stimulation by MgADP.

Recently, it was speculated that ATP hydrolysis in both nucleotide binding folds switches sulfonylurea receptors (the regulatory subunits of K_{ATP} channels) from an „off“ into an „on“ conformation, which besides high affinity for potassium channel openers and nucleoside diphosphates is characterised by a slightly reduced affinity for sulfonylureas. In this model PHHI results from locking the receptor in its „off“-conformation, which should result in loss of KCO binding and lack of an inhibitory effect of MgATP on glibenclamide binding.

To test this hypothesis 22 mutations in SUR1 known to cause PHHI were functionally analyzed. Nine of the constructs did not show any high affinity glibenclamide binding suggesting that the mutations did either induce truncation or incorrect folding of the protein resulting in a complete loss of channel activity. Among the mutations with preserved high affinity binding of [^{3}H]glibenclamide four were found to completely abolish and nine to significantly weaken diazoxide binding. Interestingly, the mutations did also lead to either an abolishment or a strong reduction of the inhibitory effect of 100 µM MgATP on high affinity [^{3}H]glibenclamide binding. Indeed, the effects of all mutations on these parameters were strikingly parallel evidenced by the fact that the square of diazoxide binding showed a strong linear correlation with the effect of ATP on glibenclamide binding

These findings thus convincingly support the idea that locking the receptor in its non-binding state induces PHHI via preventing K_{ATP} channel activation by physiological activators, specifically MgADP.

Interestingly, PHHI mutations resulting in loss of potassium channel opener binding were not only localized in NBF1 or NBF2 but also in areas outside the nucleotide binding folds, particularly in transmembrane domains 3, 8, 9 and 11 and the intracellular loops 4 and 8, suggesting that these regions are involved in the switching process.

Recently, the three-dimensional crystal structure of HisP, the ATP binding subunit of the histidine permease, has been elucidated. Sequence alignment of HisP with the NBFs of SUR1 revealed a high degree of homology and suggested very similar tertiary structures. Based on this alignment and the data from the crystal structure, a tertiary structure of the NBFs of SURs was deduced and the functional relevance of residues predicted to be critical for ATP binding and/or hydrolysis was examined. As expected, substitution of highly conserved amino acids in both NBFs involved in stabilizing the orientation of ATP by interacting directly with the β-phosphate or indirectly via Mg^{2+} or hydrogen bonding with the γ-phosphate induced a partial or complete loss of diazoxide binding.

To propose a structural basis for the defects of PHHI mutants inducing partial or complete loss of diazoxide binding, the location of several PHHI mutations in the tertiary structure was determined. The results suggest that some of the PHHI mutants (e.g. R1353P or S1387F) are critical for the coordination of ATP, while others (e.g. R1437Q, G1479R) are essential for transducing conformational changes resulting from ATP hydrolysis.

Thus in this last part of the thesis, evidence is presented that the tertiary structure of the NBFs of SURs closely resembles that of HisP and allows a predicition of the functional consequences of mutations leading to a dysregulation of insulin secretion.

7. References

ABEL K, YODER MD, HILGENFELD R, JURNAK F (1996): An alpha to beta conformational switch in EF-Tu. *Structure* **4**, 1153 - 1159.

AGUILAR-BRYAN L, NICHOLS CG, WECHSLER SW, CLEMENT JP IV, BOYD AE III, GONZALEZ G, HERRERA-SOSA H, NGUY K, BRYAN J, NELSON DA (1995): Cloning of the β cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science* **268**, 423 - 426.

AGUILAR-BRYAN L, BRYAN J (1996): ATP-sensitive potassium channels, sulfonylurea receptors and persistent hyperinsulinemic hypoglycemia of infancy. *Diabetes Rev* **4**, 336 - 346.

AGUILAR-BRYAN L, CLEMENT JP IV, GONZALEZ G, KUNJILWAR K, BABENKO A, BRYAN J, (1998): Toward understanding of the assembly and structure of K_{ATP}-channels. *Physiol Rev* **78,** 227 - 245.

AGUILAR-BRYAN L, BRYAN J (1999): Molecular biology of adenosine triphosphate-sensitive potassium channels. *Endocr Rev* **20**, 101 – 135.

AMES GF-L, LECAR H (1992): ATP-dependent bacterial transporters and cystic fibrosis: Analogy beween channels and transporters. *FASEB J* **6**, 2660 – 2666.

ANDERSON MP, WELSH MJ (1992): Regulation by ATP and ADP of CFTR chloride channels that contain mutant nucleotide-binding domains. *Science* **257**, 1701 - 1703.

ASHCROFT FM (1988): Adenosine 5´-triphosphate-sensitive potassium channels. *Annu Rev Neurosci* **11**, 97 - 118.

ASHCROFT FM, RORSMAN P (1991): Electrophysiology of the pancreatic β-cell. *Prog Biophys molec Biol* **54**, 87 - 143.

ASHCROFT FM, GRIBBLE FM (1999): ATP-sensitive K^+ channels and insulin secretion: their role in health and disease. *Diabetologia* **42**, (8):903-19.

ASHCROFT SJH, ASHCROFT FM (1990): Properties and functions of ATP-sensitive K^+channels. *Cell signal* **2**, 197 - 214.

ASHCROFT SJH, ASHCROFT FM (1992): The sulfonylurea receptor. *Biochem Biophys Acta* **1175**, 45 - 59.

AUCHAMPACH JA, CAVERO I, GROSS GJ (1992): Nicorandil attenuates myocardial dysfunction associated with transient ischemia by opening ATP-dependent potassium channels. *J Cardiovasc Pharmacol* **20**, 765-771.

AUCHAMPACH JA, MARUYAMA M, GROSS GJ (1994): Cardioprotective actions of potassium channel openers. *Eur Heart J* **15**, 89-94.

AUMÜLLER W, BÄNDER A, HEERDT R (1966): Ein neues, hochwirksames Antidiabeticum. *Arzneimittel-Forsch* **16**, 1640 – 1641.

AYNSLEY-GREEN A (1981): Nesidioblastosis of the pancreas in infancy. *Dev. Med. Child Neurol* **23**, 372-379.

BEAUDET L, GROS P (1995): Functional dissection of P-glycoprotein nucleotide-binding domains in chimeric and mutant proteins. *J Biol Chem* **270**, 17159 - 17170.

BEECH DJ, ZHANG H, NAKAO K, BOLTON TB (1993): K channel activation by nucleotide diphosphates and its inhibition by glibenclamide in vascular smooth muscle cells. *Br J Pharmacol* **110**, 573 - 582.

BENNETT JP (1978): Methods in binding studies. In: Yamamura HI, Enna SJ and Kuhar MJ, *Neurotransmitter Receptor Binding*; Raven Press, New York 1978: 57 - 90.

BRAY KM , QUAST U (1992): A specific binding site for K^+-channel openers in rat aorta. *J Biol Chem* **267**, 11689 - 11692.

BRUINING GJ (1990): Recent advances in hyperinsulinism and the pathogenesis of diabetes mellitus. *Curr Opin Pediatr* **2**, 758-765.

BRYAN J, AGUILAR-BRYAN L (1997): The ABCs of ATP-sensitive potassium channels: more pieces of the puzzle. *Curr Opin Cell Biol* **9**, 553 - 559.

CHENG Y-C, PRUSOFF WH (1973): Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**, 3099 - 3108.

CHUTKOW WA, SIMON MC, LE BEAU MM, BURANT CF (1996): Cloning, tissue expression and cromosomal localization of SUR2, the putative drug-binding subunit of cardiac, skeletal muscle and vascular K_{ATP} channels. *Diabetes* **45**, 1439 - 1445.

CLEMENT JP IV, KUNJILWAR K, GONZALEZ G, SCHWANSTECHER M, PANTEN U, AGUILAR-BRYAN L, BRYAN J (1997): Association and stoichiometry of K_{ATP} channel subunits. *Neuron* **18**, 827 - 838.

COETZEE WA (1992): ATP-sensitive potassium channels and myocardial ischemia: why do they open? *Cardiovasc Drugs Ther* **6**, 201 - 208.

COOK DL, HALES CN (1984): Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature* **311**, 271 - 273.

COOK DL, SATIN LS, ASHFORD MLJ, HALES CN (1988): ATP-sensitive K^+ channels in pancreatic β-cells. Spare channel hypothesis. *Diabetes* **37**, 495 - 498.

COOK NS, QUAST U (1990): Potassium channel pharmacology. In: *Potassium Channels, Structure, Classification, Function and Therapeutic Potential.* (Cook, NS et al.) Ellis Horwood, Chichester, 181 - 255.

CROOP JM (1998): Evolutionary relationships among ABC transporters. *Methods Enzymol*. **292**:101-16.

DAVIES NW, STANDEN NB, STANFIELD PR (1991): ATP-dependent potassium channels of muscle cells: their properties, regulation, and possible functions. *J Bioenerg Biomembr* **23**, 509 - 535.

DEAN PM, MATTHEWS EK (1968): Electrical activity in pancreatic islet cells. *Nature* **219**, 389 - 390.

DEBLASI A, O´REILLY K, MOTULSKY HJ (1989): Calculating receptor number from binding experiments using same compound as radioligand and competitor. *Trends Pharmacol Sci* **10**, 227 - 229.

D'HAHAN N, MOREAU C, PROST AL, JACQUET H, ALEKSEEV AE, TERZIC A, VIVAUDOU M (1999): Pharmacological plasticity of cardiac ATP-sensitive potassium channels toward diazoxide revealed by ADP. *Proc Natl Acad Sci USA* **21**, 2162-7.

DOIGE CA, AMES GF-L (1993): ATP-dependent transport systems in bacteria and humans: Relevance to cystic fibrosis and multidrug resistance. *Annu Rev Microbiol* **47**, 291 – 319.

DOLLERY CT, PENTECOST BL, SAMAAN NA (1962): Drug induced diabetes. *Lancet* **ii**, 735 - 737.

DÖRSCHNER H, BREKARDIN E, UHDE I, SCHWANSTECHER C, SCHWANSTECHER M (1999): Stoichiometry of sulfonylurea-induced ATP-sensitive potassium channel closure. *Mol Pharmacol* **55**, 1060 – 1066.

DUNNE MJ, PETERSEN OH (1986a): GTP and GTP activation of K^+ channels that are inhibited by ATP. *Pfügers Arch* **407**, 564 – 565.

DUNNE MJ, PETERSEN OH (1986b): Intracellular ADP activates K^+ channels that are inhibited by ATP in an insulin-secreting cell-line. *FEBS Lett* **208**, 59 – 62.

DUNNE MJ, KANE C, SHEPHERD RM, SANCHEZ JA, JAMES RF, JONSON PR, AYNSLEY-GREEN A, LU S, CLEMENT JP IV, LINDLEY KJ, SEINO S, AGUILAR-BRYAN L (1997): Familial persistent hyperinsulinemic hypoglycemia of infancy and mutations in the sulfonylurea receptor. *N Engl J Med* **336**, 703 - 706.

DRAIN P, LI L, WANG J (1998): K_{ATP} channel inhibition by ATP requires distinct functional domains of the cytoplasmic C terminus of the pore-forming subunit. *Proc Natl Acad Sci* USA 95, 13953 – 13958.

EDWARDS G, WESTON AH (1990): Structure-activity relationships of K^+ channel openers. *Trends Pharmacol Sci* **11**, 417 - 422.

EDWARDS G, WESTON AH (1995): Pharmacology of the potassium channel openers. *Cardiovasc Drugs Ther* **9**, 185 - 193.

ESCANDE D, THURINGER D, LE GUERN S, CAVERO I (1988): The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+-channels in isolated cardiac myocytes. *Bioch Biophys Res Comm* **154**, 620 - 625.

ESCANDE D, THURINGER D, LE GUERN S, COURTEIX J, LAVILLE M, CAVERO I (1989): Potassium channel openers act through an activation of ATP-sensitive K^+-channels in guineapig cardiac myocytes. *Pflügers Arch* **414**, 669 - 675.

ESCANDE D, CAVERO I (1992): K^+-channel openers and `natural` cardioprotection. *Trends Pharmacol Sci* **13**, 269 - 271.

FINDLAY I (1987): The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. *J Physiol* **391**, 611 - 629.

FOSSET M, DE WEILLE JR, GREEN RD, SCHMID-ANTOMARCHI H, LAZDUNSKI M (1988): Antidiabetic sulfonylureas control action potential properties in heart cells via high affinity receptors that are linked to ATP-dependent K^+ channels. *J Biol Chem* **263**, 7933 - 7936.

FRY DC, KUBY SA, MILDVAN AS (1986): ATP-binding site of adenylate kinase: Mechanistic implications of its homology with *ras*-encoded p21, F_1-ATPase, and other nucleotide-binding proteins. *Proc Natl Acad Sci USA* **83**, 907 - 911.

GAINES KL, HAMILTON S, BOYD AE III (1988): Characterization of the sulfonylurea receptor on beta-cell membranes. *J Biol Chem* **263**, 2589 - 2592.

GANDOLFO G, GOTTESMANN C, BIDARD JN, LAZDUNSKI M (1989): K^+-channel openers prevent epilepsy induced by the bee venom peptide MCD. *Eur J Pharmacol* **159**, 329 - 330.

GARRINO MG, HENQUIN JC (1987): Adamantane derivates: a new class of insulin secretagogous. *Br J Pharmacol* **90**, 583 - 591.

GEISEN K, HITZEL V, OEKOMONOPOULUS R, PÜNTER J, WEYER R, SUMM HD (1985): Inhibition of ^{3}H-glibenclamide binding to sulfonylurea receptors by oral antidiabetics. *Arzneimittelforschung* **35**, 707 - 712.

GILMAN AG, GOODMAN LS, RALL TW, MURAD F (1985): The Pharmacological Basis of Therapeutics, 7th edition. New York, MacMillan, 799 und 1512.

GLUZMAN Y (1981): SV40-transformed simian cells support the replication of early SV40 mutants.*Cell* **23**, 175 - 82.

GOPALAKRISHNAN M, JANIS RA, TRIGGLE DJ (1993): ATP-sensitive K^+ channels: Pharmacologic properties, regulation and therapeutical potential. *Drug Dev Res* **28**, 95 - 127.

GORUS FK, SCHUIT FC, IN´T VELD PA, GEPTS W, PIPELEERS DG (1988): Interaction of sulfonylureas with pancreatic β-cells. *Diabetes* **37**, 1090 - 1095.

GRANT DB, DUNGER DB, BURNS EC (1986): Long treatment with diazoxide in childhood hyperinsulinism. *Acta Endocrinologica* **279**, 340 – 345.

GRIBBLE FM, ASHFIELD R, ÄMMÄLÄ C, ASHCROFT FM (1997): Properties of cloned ATP-sensitive K^+ currents expressed in Xenopus oocytes. *J Physiol* **498**, 87 - 98.

GROSS GJ (1991): Coronary blood flow studies with potassium channel openers. In: *Current Drugs: Potassium Channel Modulators*. (Tarr L, ed) London, Current Patent Ltd, KCM-B82-B92.

GROSS GJ, AUCHAMPACH JA (1992a): Role of ATP dependent potassium channels in myocardial ischaemia. *Cardiovasc Res* **26**, 1011 - 1016.

GROSS GJ, AUCHAMPACH JA (1992b): Blockade of ATP-sensitive potassium channels prevent myocardial preconditioning in dogs. *Circ Res* **70**, 223 - 233.

GROSS I, TOMAN A, UHDE I, SCHWANSTECHER C, SCHWANSTECHER M (1999): Stoichiometry of potassium channel opener action. *Mol Pharmacol* **56**, 1370 - 1373

GROVER GJ (1991): Potassium channel opener in the treatment of myocardial ischemia. In: *Current Drugs: Potassium Channel Modulators*. (Tarr L, ed) London, Current Patent Ltd, KCM-B29-B38.

GROVER GJ (1994): Protective effects of ATP sensitive potassium channel openers in models of myocardial ischemia. *Cardiovasc Res* **28**(6), 778 - 782.

GUMERLOCK MK (1989): Cerebrovascular disease and the blood-brain barrier. In: Neuwelt EA; *Implications of the Blood-Brain Barrier and Its Manipulation, vol **2***; Plenum, New York, 495 - 565.

HAMILTON TC, WEIR SW, WESTON AH (1986): Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. *Br J Pharmacol* **88**, 103 - 111.

HARNESS JK, GEELHOED GW, THOMPSON NW (1981): Nesidioplastosis in adults: a surgical dilemma. *Archives of surgery* **116**, 575-580

HENQUIN JC, MEISSNER HP (1982): Opposite effects of tolbutamide and diazoxide on ^{86}Rb fluxes and membrane potential in pancreatic beta cells. *Biochem Pharmacol* **31**, 1407 - 1415.

HENQUIN JC (1987): Regulation of insulin release by ionic and electrical events in β-cells. *Hormone Res* **27**, 168 - 178.

HEURTEAUX C, BERTAINA V, WIDMANN C, LAZDUNSKI M (1993): K^+ channel openers prevent global ischemia-induced expression of c-fos, c-jun, heat shock protein, and amyloid β-protein precursor genes and neuronal death in rat hippocampus. *Proc Natl Acad Sci USA* **90**, 9431 - 9435.

HIGGINS CF (1992): ABC transporters: From microorganisms to man. *Annu Rev Cell Biol* **8**, 67 - 113.

HIGGINS CF (1995): The ABC of channel regulation. *Cell* **82**, 693 - 696.

HUNG LW, WANG IX, NIKAIDO K, LIU PQ, AMES GFL, KIM SH (1998): Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature* **396**, 703 – 707.

IBBOTSON T, EDWARDS G, NOACK T, WESTON AH (1993): Effects of P1060 and Aprikalim on whole cell currents in rat portal vein: inhibition by glibenclamide and phentolamine. *Br J Pharmacol* **108**, 991 - 998.

INAGAKI N, GONOI T, CLEMENT JP IV, NAMBA N, INAZAWA J, GONZALEZ G, AGUILAR-BRYAN L, SEINO S, BRYAN J (1995): Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. *Science* **270**, 1166 - 1170.

INAGAKI N, GONOI T, CLEMENT JP IV, WANG CZ, AGUILAR-BRYAN L, BRYAN J, SEINO S (1996): A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron* **16**, 1011 - 1017.

ISOMOTO S, KONDO C, YAMADA M, MATSUMOTO S, HIGASHIGUCHI O, HORIO Y, MATSUZAWA Y, KURACHI Y (1996): A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K^+ channel. *J Biol Chem* **271**, 24321 - 24324.

JIANG C, XIA Y, HADDAD GG (1992): Role of ATP-sensitive K^+ channels during anoxia: Major differences between rat (newborn and adult) and turtle neurons. *J Physiol* **448**, 599 - 612.

JONAS P, KOH D-S, KAMPE K, HERMSTEINER M, VOGEL W (1991): ATP-sensitive and Ca-activated K channels in vertebrate axons: novel links between metabolism and excitability. *Pflügers Arch* **418**, 68 - 73.

JONES TA (1985): Differaction methods for biological macromolecules, Interactive computer graphics: FRODO. *Methods Enzymology* **115**, 157 – 171.

KAJIOKA S, KITAMURA K, KURIYAMA H (1991): Guanosine diphosphate activates an adenosine 5´-triphosphate-sensitive K^+ channel in the rabbit portal vein. *J Physiol* **444**, 397 - 418.

KAUBISCH N, HAMMER R, WOLLHEIM C, RENOLD AE, OFFORD RE (1982): Specific receptors for sulfonylureas in brain and in B-cell tumor of the rat. *Biochem Pharmacol* **31**, 1171 - 1174.

KELLNER H-M, CHRIST O, RUPP W, HEPTNER W (1969): Resorption, Verteilung und Ausscheidung nach Gabe von ^{14}C-markiertem HB 419 an Kaninchen, Ratten und Hunde. *Arzneimittel-Forsch* **19**, 1388 - 1400.

KOLB KH, SCHULZE PE, SPECK U, ACKSTEINER B (1974): Pharmakokinetik von radioaktiv markiertem Glisoxepid beim Tier. *Arzneimittel-Forsch* **24**, 397 - 403.

KOVACS RJ, NELSON MT (1991): ATP-sensitive K^+ channels from aortic smooth muscle incorporated into planar lipid bilayers. *Am J Physiol* **261**, H604 - H609..

KRAULIS PJ (1991):MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallog* **24**, 964 – 950.

KUROIWA T, SHIBUTANI M, OKEDA R (1988): Blood-brain barrier disruption and exacerbation of ischemic brain edema after restoration of blood flow in experimental focal cerebral ischemia. *Acta Neuropathol* **76**, 62 - 70.

LANDAU H, PERLMAN M, MEYER S, ISACSONN M, KRAUSZ M, MAYAN H, LIJOVETSKY G; SCILLER M (1982): Persistent hyperglycemia due to hyperinsulinism, medical aspects. *Pediatrics* **70**, 440-446

LANDAU H, SHILLER M (1991): Persistent hyperinsulinemic hypoglycemia of infancy and childhood. In*:* Schiller M, *pediatric surgery of the liver pancreas and spleen.*; Ed. philadelphia, Saunders, 187 - 201

LAWSON K (1996a): Is there a therapeutic future for potassium channel openers? *Clinical Science* **91**, 651 - 663.

LAWSON K (1996b): Potassium channel activation: a potential therapeutic approach *Pharmacol Ther* **70**, 39 - 63.

LIGHT PE, COMTOIS AS, RENAUD JM (1994): The effect of glibenclamide on frog skeletal muscle: evidence for K_{ATP} channel activation during fatigue. *J Physiol* **475**, 495 - 507.

LIU P-Q, SHAROM FJ (1997): Fluorescence studies on the nucleotide binding domains of the P-glycoprotein multidrug transporter. *Biochemistry* **36**, 2836 – 2843.

LYNCH JJ, SANGUINETTI MC, KIMURA S, BASSETT AL (1992): Therapeutic potential of modulating potassium currents in the diseased myocardium. *FASEB J* **6**, 2952 - 2960.

MALAISSE WJ, MALAISSE-LAGAE F, MAYHEW DA, WRIGHT PH (1967): Effects of sulfonylureas upon insulin secretion by the rat´s pancreas. In: Butterfield WJH, Westering W; *Tolbutamide after ten years*; Excerpta Medica, Amsterdam, 49 - 60.

MARKWORTH E, SCHWANSTECHER C, SCHWANSTECHER M (2000): ATP4-mediates closure of pancreatic beta-cell ATP-sensitive potassium channels by interaction with 1 of 4 identical sites. *Diabetes* **49,** 1413 - 8.

MARTIN F, PINTOR J, ROVIRA JM, RIPOLL C, MIRAS-PORTUGAL MT, SORIA B (1998): Intracellular diadenosine polyphosphates: A novel second messenger in a stimulus secretion coupling. *FASEB* **12**, 1499 – 1506.

MATSUO M, TRAPP S, TANIZAWA Y, KIOKA N, AMACHI T, OKA Y, ASHCROFT FM, UEDA K **(**2000): Functional analysis of a mutant sulfonylurea receptor, SUR1-R1420C, that is responsible for persistent hyperinsulinemic hypoglycemia of infancy. *J Biol Chem* **52,** 41184 – 91.

MATTHEWS EK (1985): Electrophysiology of pancreatic islet β-cells. In: Poisner AM, Trifaro JM, *The Electrophysiol-gy of the Secretory Cell*; Elsevier, Amsterdam, 93 - 112.

MATTHEW PM; YOUNG YM, ABU-OSBA YK, MULHERN BD, HAMDAN JA, SADI AR (1988): Perisistent neonatal hyperinsulinism. *Clin Peadiat* **27**, 148-151.

MIMURA CS, HOLBROOK SR, AMES GF-L (1991): Structural model of the nucleotide-binding conserved component of periplasmic permeases. *Proc Natl Acad Sci USA* **88**, 84 - 88.

MISLER S, FALKE LC, GILLIS K, MCDANIEL ML (1986): A metabolite-regulated potassium channel in rat pancreatic B cells. *Proc Natl Acad Sci USA* **83**, 7119 - 7123.

NELSON MT, QUALYE JM (1995): Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* **268**(4), C799 - C822.

NESTOROWICZ A, INGAKI N, GONOI T, SCHOOR KP, WILSON BA, GLASER B, LANDAU H, STANLEY CA, THORNTON PS, SEINO S, PERMUTT MA (1997): A nonsense mutation in the inward rectifier potassium channel gene, Kir6,2 is associated with familial hyperinsulinism. *Diabetes* **46**, 1743 – 1748.

NESTOROWICZ A, GLASER B, WILSON BA, SHYNG SL, NICHOLAS CG, STANLEY CA, THORNTON PS, PERMUTT MA (1998): Genetic heterogeneity in familial hyperinsulinism. *Hum Mol Genet* **7**, 1119 – 1128.

NICHOLS CG, LEDERER WJ (1991): Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am J Physiol* **261**, H1675 - H1686

NICHOLS CG, SHYNG SL, NESTORWICZ A, GLASER B, CLEMENT JP, GONZALEZ G, AGUILAR-BRYAN L , PERMUTT MA, BRYAN J (1996) Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science* **272**, 1785 – 1787.

NOMA A (1983): ATP-regulated K^+ channels in cardiac muscle. *Nature* **305**, 147 - 148.

OHNO-SHOSAKU T, YAMAMOTO C (1992): Identification of an ATP-sensitive K^+-channel in rat cultured cortical neurons. *Pflügers Arch* **422**, 260 - 266.

OKUYAMA Y, YAMADA M, KONDO C, SATOH E, ISOMOTO S, SHINDO T, HORIO Y, KITAKAZE M, HORI M, KURACHI Y (1998): The effects of nucleotides and potassium channel openers on the SUR2A/Kir6.2 complex K^+-channel expressed in a mammalian cell line, HEK293T. *Pflügers Arch* **435**, 595 - 603.

OTONKOSKI T, ÄMMÄLÄ C, HUOPIO H, COTE GJ, CHAPMAN JC, COSGROVE KE, ASHFIELD R, HUANG E, KOMUIANINAN J, ASCHCROFT FM, DUNNE MJ, KERE J, THOMAS PM (1999):: A point mutation inactivating the sulphonylurea receptor causes the severe form of persistent hyperinsulinemic hypoglycemia of infancy in Finland. *Diabetes* **48**, 408 - 415.

PANTEN U, BURGFELD J, GOERKE F, RENNICKE M, SCHWANSTECHER M, WALLASCH A, ZÜNKLER BJ, LENZEN S (1989): Control of insulin secretion by sulfonylureas, meglitinide and diazoxide in relation to their binding to the sulfonylurea receptor in pancreatic islets. *Biochem Pharmacol* **38**, 1217 - 1229.

PANTEN U, HEIPEL C, ROSENBERGER F, SCHEFFER K, ZÜNKLER BJ, SCHWANSTECHER C (1990): Tolbutamide-sensitivity of the adenosine-5´-triphosphate-dependent K^+ channel in mouse pancreatic B-cells. *Naunyn-Schmiedeberg´s Arch Pharmacol* **342**, 566 - 574.

PANTEN U, SCHWANSTECHER M, SCHWANSTECHER C (1992): Pancreatic and extrapancreatic sulfonylurea receptors. *Horm Metabol Res* **24**, 549 - 554.

PETERSEN HJ, NIELSEN CK, ARRIGONI-MARTELLI E (1978): Synthesis and hypotensive activity of N-alkyl-N´´-cyano-N´-pyridylguanidines. *J Med Chem* **21**, 773 - 781.

PLANT TD, GARRINO MG, HENQUIN JC (1989): Comparison of the effects of putative activators of K^+-channels in pancreatic B-cell function. *Pflügers Arch* **414**, 152 - 153.

POLITI DMT, ROGAWSKI MA (1991): Glyburide-sensitive K^+ channels in cultured rat hippocampal neurons: Activation by cromakalim and energy-depleting conditions. *Mol Pharmacol* **40**, 308 - 315.

PORTE D, JR (1968): Inhibition of insulin release by diazoxide and its relation to catecholamine effect in man. *Ann NY Acad Sci* **150**, 281 - 286.

QUAST U, COOK NS (1989): Moving together: K^+-channel openers and ATP-sensitive K^+channels. *Trends Pharmacol Sci* **10**, 431 - 435.

QUAST U (1992): Potassium channel openers: pharmacological and clinical aspects. *Fundam Clin Pharmacol* **6**, 279 - 293.

QUAST U (1996): ATP-sensitive K^+-channels in the kidney. *Naunyn-Schmiedeberg´s Arch Pharmacol* **354**, 213 - 225.

RANDALL M, GRIFFITH T (1992): Effects of BRL 38227, sodium nitroprusside and verapamil on collateral perfusion following arterial occlusion in the rabbit isolated ear. *Br J Pharmacol* **106**, 315 - 323.

REPKE H, LIEBMANN C (1987): Membranrezeptoren und ihre Effektorsysteme. *VCH Verlagsgesellschaft mbH*, Weinheim, 1987.

RIBALET B, CIANI S (1987): Regulation by cell metabolism and adenine nucleotides of a K channel in insulin-secreting b cells (RIN m5F). *Pros Natl Acad Sci* **84**, 1721 – 1725.

ROBERTSON DW, STEINBERG MI (1990): Potassium channel modulators: Scientific application and therapeutic promise. *J Med Chem* **33**, 1529 - 1541.

SARASTE M, SIBBALD PR, WITTINGHOFER A (1990): The P-loop- A common motif in ATP- and GTP-binding proteins. *Trends Biochem Sci* **15**, 430 - 434.

SCHMID-ANTOMARCHI H, AMOROSO S, FOSSET M, LAZDUNSKI M (1990): K^+-channel openers activate brain sulfonylurea-sensitive K^+-channels and lock neurosecretion. *Proc Natl Acad Sci* USA **87**, 3489 - 3492.

SCHMID-ANTOMARCHI H, DE WEILLE J, FOSSET M, LAZDUNSKI M (1987): The rezeptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+-channel in insulin-secreting cells. *J Biol Chem* **262**, 15840 - 15844.

SCHNEIDER E, HUNKE S (1998): ATP-binding cassette (ABC) transport systems: Functional and structural aspects of the ATP-hydrolysing subunits/domains. FEMS Microbial. Rev. **22**, 1 – 20.

SCHOMBURG D, REICHELT J (1988): Bragi: A comprehensive protein modeling program system. *J Mol Graph* **6**, 161 – 165.

SCHWANSTECHER C, DICKEL C, EBERS I, LINS S, ZÜNKLER BJ, PANTEN U (1992a): Diazoxide-sensitivity of the adenosine-5´-triphosphate-dependent K^+ channel in mouse pancreatic β-cells. *Br J Pharmacol* **107**, 87 - 94.

SCHWANSTECHER C, PANTEN U (1994): Identification of an ATP-sensitive K^+ channel in spiny neurons of rat caudate nucleus. *Pflügers Arch* **427**, 187 - 189.

SCHWANSTECHER C, BASSEN D (1997): K_{ATP}-channel on the somata of spiny neurones in rat caudate nucleus: regulation by drugs and nucleotides. *Br J Pharmacol* **121** (2), 193 - 198.

SCHWANSTECHER M, LÖSER S, RIETZE I, PANTEN U (1990): Mg^{2+} ATP controls glibenclamide- and diazoxide-binding to their receptor in pancreatic B-cells. *Diabetologia* **33**, A78.

SCHWANSTECHER M, RIETZE I (1990): Hydrolyzable nucleotides inhibit glibenclamide binding in pancreatic islets. *Naunyn-Schmiedeberg´s Arch Pharmacol* **341** (suppl), R72.

SCHWANSTECHER M, BEHRENDS S, PANTEN U (1991a): The Mg^{2+}-complex of ATP regulates the affinity of the solubilized sulfonylurea receptor from HIT-cells. *Diabetologia* **34** (suppl. 2), A63.

SCHWANSTECHER M, LÖSER S, RIETZE I, PANTEN U (1991b): Phosphate and thiophosphate group donating adenine and guanine nucleotides inhibit glibenclamide binding to membranes from pancreatic islets. *Naunyn-Schmiedeberg´s Arch Pharmacol* **343**, 83 - 89.

SCHWANSTECHER M, BEHRENDS S, BRANDT C, PANTEN U (1992b): The binding properties of the solubilized sulfonylurea receptor from a pancreatic B-cell line are modulated by the Mg^{++}-complex of ATP. *J Pharmacol Exp Ther* **262**, 495 - 502.

SCHWANSTECHER M, BRANDT C, BEHRENDS S, SCHAUPP U, PANTEN U (1992c): Effects of MgATP on pinacidil-induced displacement of glibenclamide from the sulphonylurea receptor in a pancreatic β-cell line and rat cerebral cortex. *Br J Pharmacol* **106**, 295 - 301.

SCHWANSTECHER M, LÖSER S, BRANDT C, SCHEFFER K, ROSENBERGER F, PANTEN U (1992d): Adenine nucleotide-induced inhibition of binding of sulphonylureas to their receptor in pancreatis islets. *Br J Pharmacol* **105**, 531 - 534.

SCHWANSTECHER M (1994): Charakterisierung des Sulfonylharnstoffrezeptors in insulinsezernierenden Zellen und cerebralem Cortex. *Habilitationsschrift, Universität Göttingen.*

SCHWANSTECHER M (1998): Molekulare Pathophysiologie der persistierenden hyperinsulinämischen Hypoglykämie des Kleinkindesalters. In: Rabl M, Mohnike K, *"Nesidioblastose": von der molekularen Pathophysiologie zur Therapie*; Johann Ambobius Barth Verlag, Heidelberg-Leipzig,

SCHWANSTECHER M, SIEVERDING C, DÖRSCHNER H, GROSS I, AGUILAR-BRYAN L, SCHWANSTECHER C, BRYAN J (1998): Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. *EMBO J* **17,** 5529 - 5535.

SENIOR AE, GADSBY DC (1997): ATP hydrolysis cycles and mechanism in P-glycoprotein and CFTR. *Semin Cancer Biol* **3,** 143 – 50.

SELZER HS, ALLEN EW (1965): Inhibition of insulin secretion in `Diazoxide Diabetes´. *Diabetes* **14**, 439.

SHARMA N, CRANE A, GONZALEZ G, BRAYAN J, AGUILAR-BRYAN L (2000): Familial hyperinsulinism and pancreatic β-cell ATP-senitive potassium channels. *Kidney International* **57**, 803-808.

SHEN H, YAO BY, MUELLER DM (1994): Primary structural constraints of P-loop of mitochondrial F1-ATPase from yeast. *J Biol Chem*, **269,** 9424 –9428.

SHEN WK, TUNG RT, MACHULDA MM, KURACHI Y (1991): Essential role of nucleotide diphosphates in nicorandil-mediated activation of cardiac ATP-sensitive K^+-channel. *Circ Res* **69**, 1152 - 1158.

SHYAMALA V, BAICHWAL V, BEALL E, AMES GF-L (1991): Structure-function analysis of the histidine permease and comparison with cystic fibrosis mutations. *J Biol Chem* **266**, 18714 - 18719.

SHYNG SL, FERRIGNI T, NICHOLS CG (1997): Control of rectification and gating of cloned K_{ATP} channels by the Kir6.2 subunit. *J Gen Physiol* **110**, 141 - 153.

SHYNG SL, FERRIGNI T, SHEPHARD JB, SHYNG SL, NESTOROWICZ A, GLASER B, PERMUTT MA, NICHOLS CG (1998): Functional analysis of novel mutations in the sulfonylurea receptor 1 associated with persistent hyperinsulinemic hypoglycemia of infancy. *Diabetes* **47**: 1145-1151

SIEVERDING A (1998a): Regulation der hochaffinen Bindung von Sulfonylharnstoffen an SUR1 durch Nucleotide und Nucleotidanaloga. Dissertation, Technische Universität Braunschweig

SIEVERDING C (1998b): Identifikation und Charakterisierung der Bindungsstelle für Kalium-Kanal-Öffner auf Sulfonylharnstoffrezeptoren. Dissertation, Technische Universität Braunschweig

SPRUCE AE, STANDEN NB, STANFIELD PR (1985): Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature* **316**, 736 - 738.

SPRUCE AE, STANDEN NB, STANFIELD PR (1987): Studies of the unitary properties of adenosine-5´-triphosphate-regulated potassium channels of frog skeletal muscle. *J Physiol* **382**, 213 - 236.

SUGITA O, SAWADA Y, SUGIYAMA Y, IGA T, HANANO M (1982): Physiologically based pharmacokinetics of drug-drug interaction: a study of tolbutamide-sulfonamide interaction in rats. *J Pharmacokinet Biopharm* **10**, 297 - 316.

STANLEY CA, BAKER L (1976): Hyperinsulinism in infants and children, diagnosis and therapy. *Adv Pediatr* **23**, 315 - 355

TALBACHNIK D, GULBENKIAN A (1968): Mechanism of diazoxide hyperglycaemia in animals. *Ann NV Acad Sci* **150**, 204 - 218.

THOMAS PM, COTE GJ, WOHLLK N, HADDAD B, MATHEW PM, RABL W, AGUILAR-BRYAN L, GAGEL RF, BRYAN J (1995): Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycemia of infancy. *Science* **268**, 426 - 429.

THOMAS PM, WOHLLK N, HUANG E, KUHNLE U, RABL W, GAGEL RF, COTE GJ (1996a): Inactivation of the first nucleotide-binding fold of the sulfonylurea receptor, and familial persistent hyperinsulinemic hypoglycemia of infancy. *Am J Hum Genet* **59**, 510 - 518.

THOMAS PM, YE Y, LIGHTNER E (1996b): Mutations of the pancreatic islet inward rectifier Kir6.2 also leads to familial persistent hyperinsulinemic hypoglycemia of infancy. *Hum Mol Genet* **5**, 1809 - 1812.

THURINGER D, ESCANDE D (1989): Apparent competition between ATP and the potassium channel opener, RP 49356, on ATP-sensitive K^+-channels of cardiac myocytes. *Mol Pharmacol* **36**, 897 - 902.

TRUBE G, RORSMAN P, OHNO-SHOSAKU T (1986): Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β-cells. *Pflügers Arch* **407**, 493 - 499.

TUCKER SJ, GRIBBLE FM, ZHAO C, TRAPP S, ASHCROFT FM (1997): Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature* **387**, 179 - 183.

TUSNADY GE, BAKOS E, VÁRADI A, SARKADI B (1997): Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. *FEBS Lett* **402**, 1 - 3.

UHDE I, TOMAN A, GROSS I, SCWANSTECHER C, SCHWANSTECHER M (1999): Identification of the potassium channel opener site on sulfonylurea receptors. *J Biol Chem* **274**, 28079 – 28082.

WALKER JE, SARASTE M, RUNSWICK MJ, GAY NJ (1982): Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1**, 945 - 951.

WALLENSTEIN S, ZUCKER CL, FLEISS JL (1980): Some statistical methods useful in circulation research. *Circ Res* **47**, 1 - 9.

WESTON AH, EDWARDS G (1992): Recent progress in potassium channel opener pharmacology. *Biochem Pharmacol* **43**, 47 - 54.

WILDE AAM, JANSE MJ (1994): Electrophysiological effects of ATP-sensitive potassium channel modulation: implications for arrhythmogenesis. *Cardiovasc Res* **28**, 16 - 24.

WILLIAMS AJ, LEE TH, COCHRANE GM, HOPKIRK A, VYSE T, CHIEW F, LAVENDER E, RICHARDS DH, OWEN S, STONE P, CHURCH S, WOODCOCK AA (1990): Attenuation of nocturnal asthma by cromakalim. *Lancet* **336**, 334 - 336.

WITTINGHOFER A, PAI EF (1991): The structure of Ras protein: a model for a universal molecular switch. *Trends Biochem Sci* **16**, 382 - 387.

WOHL AJ (1970): Electronic molecular pharmacology: The benzothiadiazine antihypertensive agents. *Mol Pharmacol* **6**, 189 - 194.

XU X, LEE KS (1994): Characterization of ATP-inhibited K^+ current in canine coronary smooth muscle cells. *Pflügers Arch* **427**, 110 - 120.

YAMADA M, ISOMOTO S, MATSUMOTO S, KONDO C, SHINDO T,HORIO Y, KURACHI Y (1997): Sulfonylurea receptor 2B and Kir6.1 form a sulfonylurea-sensitive but ATP-insensitive K^+channel. *J Phsyiol* **499**, 715 - 720.

ZINI S, BEN-ARI Y, ASHFORD MLJ (1991): Characterization of sulfonylurea receptors and the action of potassium channel openers on cholinergic neurotransmission in guinea pig isolated small intestine. *J Pharmacol Exper Ther* **259**, 566 - 573.